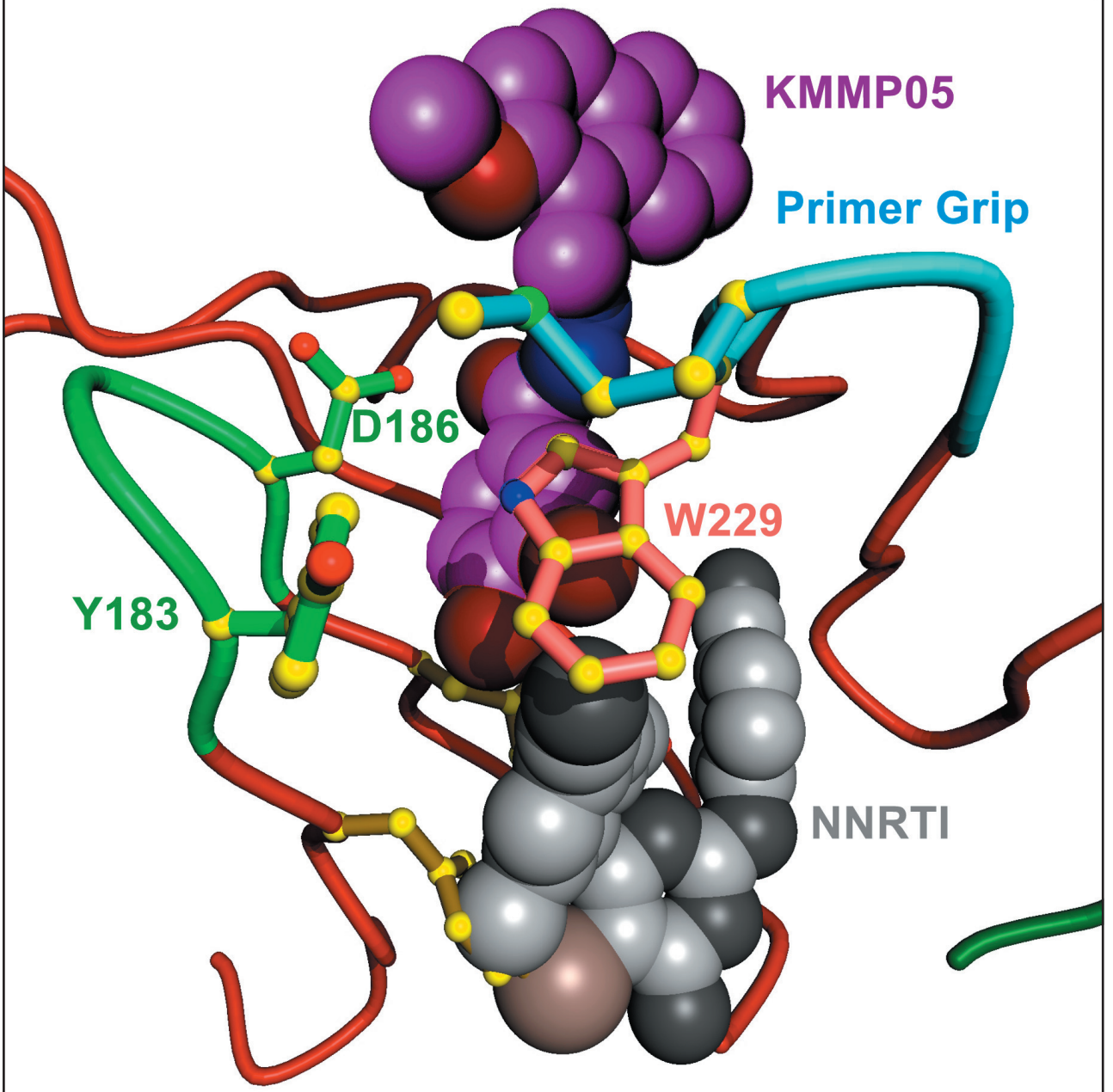
Targets and Mechanisms

6th Annual Symposium on Antiviral Drug Resistance



Program and Abstracts

November 13-16, 2005

Westfields Conference Center
Chantilly, Virginia

Sponsored by the HIV Drug Resistance Program, NCI
Co-sponsored by the University of Pittsburgh

On the cover:

Ribbon diagram of the structure of HIV-1 reverse transcriptase (RT) in complex with KMMP05 (magenta), a specific inhibitor of the RNase H activity of the enzyme. The inhibitor binds at a site that is distal to the RNase H active site and in proximity to both the polymerase active site (YMDD motif, green) and the binding pocket for nonnucleoside RT inhibitors (NNRTI, gray). The NNRTI was not present in the crystal structure of the RT-KMMP05 complex but is included in the figure to highlight the binding site of NNRTIs. This figure was provided by Daniel Himmel and Stefan Sarafianos (Center for Advanced Biotechnology and Medicine, Rutgers University); additional details of the study are provided in the abstract on page 25.

Targets and Mechanisms

6th Annual Symposium on Antiviral Drug Resistance

Sunday, November 13 – Wednesday, November 16, 2005

Symposium Organizers

John M. Coffin, Ph.D.
HIV Drug Resistance Program
National Cancer Institute

John W. Mellors, M.D.
Division of Infectious Diseases
University of Pittsburgh

Sunday	6:00 pm	Session 1: Introduction and Overviews
	7:35 pm	Reception and Dinner
Monday	9:00 am	Session 2: Biology of Viral Infection
	12:00 pm	Lunch
	1:00 pm	Session 2 (cont'd)
	2:10 pm	Session 3: Viral Entry
	5:30 pm	Poster Session and Reception
	7:00 pm	Dinner
Tuesday	8:30 am	Session 4: Nucleic Acid Replication
	12:00 pm	Lunch
	1:00 pm	Session 4 (cont'd)
	3:20 pm	Session 5: Novel Antiviral Strategies
	5:40 pm	Poster Session and Reception
	7:00 pm	Dinner
Wednesday	8:30 am	Session 6: Assembly, Release, and Processing
	12:10 pm	Lunch and Adjournment

Oral sessions are located in the *Lincoln Forum Amphitheater*

Poster sessions are located in the *Washingtonian Room*

All meals are in the *Fairfax Room;* on Monday through Wednesday, breakfast is served from 7:00 to 8:30 am

Please note: Use of recording devices (including cameras, video, and tape recorders) by participants is not permitted during the oral and poster sessions.

Program at a Glance

FOREWORD

Effective antiviral therapy is the only hope for survival or alleviation of disease for millions of Americans and tens of millions of individuals worldwide suffering from chronic viral infection, including those caused by HIV, HBV, HCV, and others. In addition, the threat of global outbreaks continues from influenza, SARS, and other respiratory viruses. Despite considerable progress in the development of effective inhibitors directed at specific aspects of viral life cycles, therapeutic efficacy has been limited by the evolution of resistant virus. This problem not only results in the failure of therapy, but may limit the effectiveness of subsequent therapies. Moreover, attempts to counter drug resistance lead to complex, expensive, and toxic regimens. Antiviral drug resistance is therefore of paramount importance in dealing with growing epidemics of virus infection.

The Symposium on Antiviral Drug Resistance brings together researchers in a variety of virus systems to exchange new information on viral targets for therapy, on antiviral drugs, and on resistance to these drugs. We believe that understanding the similarities and differences of the diverse viral systems will lead to new insights into the problem of resistance in each individual virus. This year we have added the subtitle “Targets and Mechanisms” to emphasize these areas in the scope of the Symposium.

The Symposium is sponsored by the HIV Drug Resistance Program (HIV DRP) and co-sponsored by the University of Pittsburgh. The HIV DRP was established in 1997 as part of the ongoing HIV/AIDS research activity in the National Cancer Institute. Centered at the NCI-Frederick Campus, the Program also includes investigators in the NIH Clinical Center and collaborators in a number of academic centers, including the University of Pittsburgh.

ACKNOWLEDGEMENTS

We thank those who helped to make the 6th Annual Symposium on Antiviral Drug Resistance: Targets and Mechanisms possible:

- The Center for Cancer Research, National Cancer Institute, for providing the primary financial support
- The University of Pittsburgh for co-sponsorship
- Members of the HIV Drug Resistance Program for assistance and advice in many areas, in particular Anne Arthur, who devoted many long hours to mailing lists, website design and implementation, program assembly, and countless other details without which this meeting could not have happened
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- The following corporate sponsors for providing additional financial support:

6TH ANNUAL SYMPOSIUM ON ANTIVIRAL DRUG RESISTANCE: TARGETS AND MECHANISMS

Program[a]

SUNDAY, NOVEMBER 13, 6:00 – 7:35 PM

Session 1 Introduction and Overviews

Chair: **John Mellors,** Division of Infectious Diseases, University of Pittsburgh School of Medicine, Pittsburgh, PA

J.N. Strathern, Center for Cancer Research, National Cancer Institute, NCI-Frederick, Frederick, MD: *Welcoming Remarks*

* S.B. Levy, Center for Adaptation Genetics and Drug Resistance, Tufts University School of Medicine, Boston, MA: *Antibiotic Resistance: Magnitude of the Problem* Page 1

* K.M. De Cock, CDC Kenya, Nairobi, Kenya: *Status of the HIV/AIDS Pandemic – 2005* 2

Welcome Reception and Dinner

MONDAY, NOVEMBER 14, 9:00 AM – 2:10 PM

Session 2 Biology of Viral Entry

Co-Chairs: **José Esté,** Fundació irsiCaixa, Hospital Universitari Germans Trias i Pujol, Badalona, Spain
Jonathan Stoye, MRC National Institute for Medical Research, London, United Kingdom

* J. Skehel, MRC National Institute for Medical Research, London, United Kingdom: *Haemagglutinin Receptor Binding in the Emergence of Human Influenza*

* D. Lowy[1], P. Day[1], R. Richards[1], C. Buck[1], D. Pastrana[2], R. Roden[3], and J. Schiller[1], [1]Laboratory of Cellular Oncology and [2]Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD; [3]Johns Hopkins University Medical Center, Baltimore, MD: *Prevention of Papillomavirus Infection* 3

M.R. Jordan[1], M. Kearney[2], S. Palmer[2], W.-C. Goh[1], V. Bau[1], D. Stone[1], C. Wanke[1], E. Coakley[3], C. Chappey[3], and J. Coffin[2], [1]Tufts-New England Medical Center, Boston, MA; [2]HIV Drug Resistance Program, National Cancer Institute, NCI-Frederick, Frederick, MD; [3]ViroLogic, Inc., South San Francisco, CA: *Prevalence of HIV Antiretroviral Drug Resistance Mutations in Naïve Subjects With an Analysis of* pol *Gene Sequences Derived From Cloning and From Single Genome Sequencing From Eight Individuals* 4

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[a] Keynote talks are designated with an asterisk; presenters' names are underlined.

Coffee Break

MONDAY, NOVEMBER 14, 2:10 – 5:20 PM

Session 3 Viral Entry

Chair: **Henrik Garoff,** Karolinska Institute, Huddinge, Sweden

Coffee Break

MONDAY, NOVEMBER 14, 5:30 – 7:00 PM

POSTER SESSION AND RECEPTION

Posters in this session will also be displayed on November 15 (see abstracts on pages 45-87).

TUESDAY, NOVEMBER 15, 8:30 AM – 2:50 PM

Session 4 Nucleic Acid Replication

Co-Chairs: **Ellie Ehrenfeld,** National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD
Richard D'Aquila, Vanderbilt University School of Medicine, Nashville, TN

TUESDAY, NOVEMBER 15, 5:40 – 7:00 PM

POSTER SESSION AND RECEPTION

Posters in this session will also be displayed on November 14 (see abstracts on pages 45-87).

WEDNESDAY, NOVEMBER 16, 8:30 AM – 12:00 NOON

Session 6 Assembly, Release, and Processing

Chair: **Celia Schiffer,** University of Massachusetts Medical School, Worcester, MA

Coffee Break

POSTER PRESENTATIONS

AUTHOR INDEX [a]

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[a] Numbers in boldface indicate presenting authors.

ANTIBIOTIC RESISTANCE: MAGNITUDE OF THE PROBLEM

Stuart B. Levy, M.D.

Center for Adaptation Genetics and Drug Resistance, Tufts University School of Medicine, Boston, MA 02111

Bacterial resistance to antibiotics plagues patients and health care providers in countries worldwide. The prior decades of antibiotic misuse have led to the emergence of microorganisms bearing resistance to multiple antibiotics in hospitals and communities. In some patients, the infecting strain may be resistant to all available antibiotics. New resistant forms of well-known pathogens, like *S. aureus*, pneumococci and *C. difficile* have emerged, presenting grave therapeutic challenges in communities and hospitals. The recognition that this problem is global and that microbes can pass easily from one country to another, has underscored the need for surveillance of resistance, both locally and globally.

The antibiotic resistance problem stems from the coming together of the *antibiotic* which selects and the *antibiotic resistance determinants* being selected. Interventions which reduce either factor will decrease antibiotic resistance. Lessons learned from the phenomenon of drug resistance in bacteria can be extended to similar problems involving other microorganisms, such as viruses and parasites.

The Alliance for the Prudent Use of Antibiotics (APUA), an international organization with members in over 100 countries and 50 local chapters, was established in 1981. APUA has organized a global surveillance project called GAARD (Global Advisory on Antibiotic Resistance Data) which examines the drug resistance profiles of important clinical pathogens at sites throughout the world. This public/private collaboration of five large industry-sponsored surveillance systems includes advisors from WHO and CDC. GAARD allows early detection of antimicrobial resistance such as its report of fluoroquinolone resistance among *Hemophilus influenzae*. It likewise follows trends in penicillin resistance among the pneumococci worldwide. More recently, GAARD reported on drug susceptibility of *E. coli* in different geographic sites. In August, it published in *Clinical Infectious Diseases* its global 2005 report *The Shadow Epidemic* on resistance in bacteria, viruses and parasites. A complementary APUA project, Reservoirs of Antibiotic Resistance (ROAR), funded by NIAID, studies resistance genes in non-clinical, commensal bacteria which serve as natural reservoirs of resistance traits transferable to pathogens.

Through the concerted and combined efforts of scientists from various disciplines (microbiology, molecular biology, biochemistry, genetics, epidemiology) in cooperation with public health organizations, we can achieve control and reversal of antimicrobial resistance which confronts and undermines the successful therapy of infectious diseases globally.

STATUS OF THE HIV/AIDS PANDEMIC – 2005

Kevin M. De Cock, MD

CDC Kenya

By end 2004 an estimated 39.4 million people were living with HIV worldwide, 65% of them in sub-Saharan Africa, which only accounts for approximately 10% of the world's population. This overview of the current status of the pandemic will examine the origins of HIV-1 and HIV-2; review global epidemiology, contrasting the situation in the United States and sub-Saharan Africa; discuss potential reasons for epidemiologic variation, including the role of herpes simplex type 2 infection and male circumcision; examine aspects of the global response, especially treatment scale-up and its obstacles; discuss HIV-associated tuberculosis in the antiretroviral treatment era; and conclude with some unifying questions and observations. Key challenges to containing the pandemic and its devastating impact in Africa include ensuring effective prevention in the context of treatment scale-up; appropriately addressing tuberculosis; and strengthening public health, clinical, and political infrastructure over the longer term in this most disadvantaged continent.

PREVENTION OF PAPILLOMAVIRUS INFECTION

Doug Lowy[1], Patricia Day[1], Rebecca Richards[1], Christopher Buck[1], Diana Pastrana[2], Richard Roden[3], and John Schiller[1]

[1]Laboratory of Cellular Oncology, CCR; [2]Laboratory of Molecular Biology, CCR; [3]Department of Pathology, Johns Hopkins University Medical Center

Infection by certain human papillomaviruses (HPV) causes virtually all cases of cervical cancer, which is the second most common cause of cancer deaths in women worldwide. A variable proportion of other epithelial cancers is also attributable to HPV infection. We have been studying basic and translational aspects of papillomavirus infection.

In an immunologically oriented set of experiments, we found that the viral L1 major capsid protein was able to self-assemble into virus-like particles (VLP) that are capable of inducing high titers of type-specific neutralizing antibodies in animals and in humans. In preclinical papillomavirus models, L1 VLPs protected animals against high dose challenge by the homologous animal papillomavirus. Two pharmaceutical companies, Merck and GlaxoSmithKline, are developing commercial versions of the L1 VLP vaccine, and their vaccine efficacy trials have confirmed that L1 VLP vaccines can protect women against infection with the HPV types targeted by the respective vaccines. We have recently identified a region of the viral L2 minor capsid protein that can induce neutralizing antibodies against a broad spectrum of papillomaviruses, in contrast to the predominantly type-specific neutralizing antibodies induced by L1 VLPs. We plan to explore the potential of L2 as a pan-HPV vaccine.

In other studies, we have developed a simple and efficient method for producing high titer papillomavirus pseudoviruses, which are composed of the structural viral proteins, L1 and L2, and an encapsidated reporter plasmid. The pseudoviruses mimic the entry process of authentic virus. One application of the pseudoviruses has been to use them to screen for inhibitors of HPV infection. This screen has led to the identification of three potent inhibitors: alpha-defensins, carrageenan, and inhibitors of furin, a cell encoded proprotein convertase. Two of these inhibitors – alpha-defensins and carrageenan – have been reported previously to inhibit HIV infection in vitro. As carageenan is already in clinical trials as a topical microbicide whose main goal is to inhibit HIV transmission, it might be worthwhile to consider its potential to prevent HPV transmission as well.

PREVALENCE OF HIV ANTIRETROVIRAL DRUG RESISTANCE MUTATIONS IN NAÏVE SUBJECTS WITH AN ANALYSIS OF *POL* GENE SEQUENCES DERIVED FROM CLONING AND FROM SINGLE GENOME SEQUENCING FROM EIGHT INDIVIDUALS

MR Jordan[1], M Kearney[2], S Palmer[2], W-C Goh[1], V Bau[1], D Stone[1], C Wanke[1], E Coakley[3], C Chappey[3], and J Coffin[2]

[1]Tufts-New England Medical Center, Boston MA, USA; [2]HIV Drug Resistance Program, National Cancer Institute, Frederick, MD, USA; [3]ViroLogic, Inc., South San Francisco, CA, USA

Aim: To determine the prevalence of HIV drug resistance mutations and subtypes in a cohort of antiretroviral drug naïve individuals and to determine differences in viral diversity between sequences derived from cloning and sequences derived by single genome sequencing (SGS).

Methods: Thirty-one antiretroviral naïve HIV-1 positive individuals were enrolled from the Tufts-New England Medical Center infectious disease clinic or from prison medical centers in the State of Massachusetts. The mean plasma RNA was 66,720 copies/mL; mean CD4 cell count was 612 cells/mm^3. An average of 15 clones were obtained from each of the thirty-one samples. To date, SGS has been performed on eight of the thirty-one samples using a previously described protocol; an average of 19 SGS sequences were obtained from each sample. To assess variability between clonal sequences and genomes derived by SGS, *pol* genes derived by SGS and by cloning were aligned by Clustal W. Phylogenetic analyses were performed using PHYLIP and MEGA with standard reference sequences obtained from the Los Alamos National Laboratory HIV Database. Neighbor-Joining tree construction with bootstrap re-sampling of 1000 trees was performed. The within and between group average distances (as a percent mismatch between pairs of RT sequences) were calculated using MEGA.

Results: The overall prevalence of HIV-1 resistance mutations as defined by IAS-USA guidelines was 16% by population-based sequencing and 22% by clonal analyses. The neighbor-joining tree of clonal and SGS derived sequences show an intermingling of sequences from the same patient. For two of the eight patient samples, SGS revealed greater genetic diversity as the nucleotide level than cloning.

Conclusions: The prevalence of drug resistance mutations is strikingly high but consistent with reports from other urban American centers indicative of infection with drug resistant virus. More appropriate phylogenetic analyses to better describe intrapatient HIV quasispecies are ongoing.

THE CLINICAL SIGNIFICANCE OF DRUG RESISTANCE: POPULATIONS, PATIENTS, AND POLYMERASES

Martin Markowitz, M.D.

Aaron Diamond AIDS Research Center, New York, NY

Resistance to antiretroviral therapy has been a common outcome in patients treated for HIV-1 infection. Causes are numerous and include virologic factors (high replication rates, error prone RT, chronicity of infection) as well as issues surrounding drug adherence, potency, interactions and other as yet understood variables. Given the pool of patients harboring drug resistant variants it is not surprising that transmitted resistance to antiretroviral drugs in acute and early HIV-1 has been well documented. We have performed resistance testing in a cohort study of 361 patients with acute or recent HIV-1 infection enrolled between 1995 and 2004. The prevalence of transmitted resistance increased from 13·1% during the period 1995 to 1998 to 24·1% during the period 2003 to 2004 (p=0·09). NNRTI resistance rose from 2·6% to 14·3% (p=0·04) and multidrug resistant virus also increased significantly from 2·6%, to 9·8% (p=0·01) during the periods 1995 to 1998 and 2003 to 2004, respectively. Resistance to protease inhibitors alone was relatively rare throughout the study period. We have thus employed PI-based ART as initial empiric treatment in the setting of acute infection. We are encouraged that with this approach as well as resistance test-guided therapy, an excellent and comparable immunological and virologic response has been observed regardless of baseline viral drug susceptibility. The management of chronically infected patients harboring multidrug resistant viruses remains a greater challenge. Based on studies of Deeks et al it would appear that patients are likely to maintain a virologic and immunologic benefit most from continuation of NRTI therapy compared to PIs and NNRTIs in the setting of virologic failure. Whether this is due to issues of fitness of resistant variants or other factors remains obscure. Finally we have characterized a panel of transmitted wild type and multidrug resistant viruses for growth kinetics, infectivity and cytopathicity. These data suggest that despite the many amino acid changes in polymerase that are associated with multidrug resistance, compensatory mechanisms are at play, rendering these variants transmissible and fit.

PERSISTENCE OF NNRTI-RESISTANT VARIANTS AFTER SINGLE-DOSE NEVIRAPINE

S. Palmer[1], V. Boltz[1], L. Morris[2], F. Maldarelli[1], N. Martinson[3], G. Gray[3], J. McIntyre[3], Investigators for the Trial 1413[4], M. Hopley[5], T. Kimura[6], D. Mayers[6], P. Robinson[6], J. Mellors[7], and J. Coffin[1]

[1]HIV Drug Resistance Program, NCI, NIH, Frederick, MD; [2]National Institute for Communicable Diseases, Johannesburg, South Africa; [3]Perinatal HIV Research Unit Chris Hani Baragwanath Hospital and University of the Witwatersrand, Johannesburg, South Africa; [4]Study Investigators, South Africa and [5]Boehringer Ingelheim ZA, Johannesburg, South Africa; [6]Boehringer Ingelheim Pharm, Ridgefield, CT; [7]University of Pittsburgh, Pittsburgh, PA

Background: Single-dose nevirapine (sdNVP) to prevent mother to child HIV-1 transmission (MTCT) selects nevirapine-resistant variants in 30-50% of mothers as determined by standard genotyping (population sequencing). It is not known how long these resistant variants persist because standard genotyping does not reliably detect variants comprising <25% of the virus population. To address this question, we used an allele-specific RT-PCR assay that quantifies NNRTI-resistant variants at frequencies < 0.1% to test longitudinal samples from HIV-1 subtype C infected women who received sdNVP and compared these results to women who had received sdNVP + 4 or 7 days of CBV for pMTCT (McIntyre et al., Int. AIDS Cong. 2004).

Methods: Follow-up plasma samples from women participating in two South African MTCT trials were tested by allele-specific RT-PCR for 103N and 181C. Samples were analyzed from baseline up to 12 months post-therapy from three treatment arms: sdNVP (27) or sdNVP + 4 or 7 days of CBV (11 each). Plasma HIV-1 RNA was converted to cDNA, and the target region was amplified and quantified by real-time PCR. This product was used as template for a second round of real-time PCR using discriminatory primers.

Results: Allele-specific RT-PCR detected 103N or 181C mutants in 78% of the women receiving sdNVP alone at week 6 and 83% at month 6 with mutant frequencies ranging from 0.2% to 75%. In the 12 month follow-up samples (all negative by standard genotyping) 56% had mutant variants ranging in frequency from 0.1-21%. Standard genotyping did not detect NVP resistance mutations at week 2 or 6 among women in this study who received sdNVP + 4 or 7 days of CBV. Allele-specific RT-PCR detected 103N or 181C variants in 6 of 22 (27%) of women receiving sdNVP + 4 or 7 days of CBV (mutant frequency 0.4-8%, median: 1.4%). There was no difference between the 4 and 7 day CBV arms in the proportion of women with NVP-resistant variants.

Conclusions: NNRTI-resistant variants selected by sdNVP can still be detected by allele-specific RT-PCR in the majority of women 6 months after standard genotyping becomes negative. The frequency of NNRTI-resistant mutants declines with time after sdNVP but can remain above pretreatment levels for at least one year. Short-course CBV (4 or 7 days) reduced the selection of NVP-resistant variants following sdNVP from 75% to 27% of women as determined by allele-specific RT-PCR. The impact of these low-frequency NVP-resistant variants on future treatment options is unknown.

THE SHAPE OF SIV-SPECIFIC CD8 T CELL RESPONSES: IMPLICATIONS FOR VACCINES

Daniel Douek

Vaccine Research Center, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD

Escape from adaptive T-cell immunity through transmutation of viral antigenic structure is a cardinal feature in the pathogenesis of SIV/HIV infection and a major obstacle to antiretroviral vaccine development. However, the molecular determinants of this phenomenon at the T-cell receptor (TCR)-antigen interface are unknown. Here, we show that mutational escape is intimately linked to the structural configuration of constituent TCR clonotypes within virus-specific T-cell populations. Analysis of SIV-specific TCR sequences revealed that polyclonal T-cell populations characterized by highly conserved TCRB CDR3 motifs were rendered ineffectual by single residue mutations in the cognate viral epitope. Conversely, diverse clonotypic repertoires without discernible motifs were not associated with viral escape. Thus, fundamental differences in the mode of antigen engagement direct the pattern of adaptive viral evolution. These findings have profound implications for the development of vaccines that elicit T-cell immunity to combat pathogens with unstable genomes.

SELECTIVE ADVANTAGE OF LTR OF HIV-1 SUBTYPE C OVER THAT OF SUBTYPE B IN IN VIVO RECOMBINATION EVENT

Yutaka Takebe

AIDS Research Center, National Institute of Infectious Diseases

We previously identified a unique geographical “hotspot” of extensive HIV-1 intersubtype recombination in western Yunnan Province (Dehong) of China, where the diverse forms of HIV-1 recombinants between subtype C and subtype B' (Thailand variant of subtype B) appear to be arising continuously. Taking this unique opportunity, we aimed to characterize their recombinant structure and to analyze the mechanism acting on in vivo recombination events between HIV-1 subtypes B' and C. Recombination breakpoint analysis based on near full-length sequences of 16 Dehong HIV-1 isolates revealed that 63% (10 of 16) were unique recombinant forms (URFs) comprised of subtypes B' and C with distinct profiles of recombination breakpoints. HIV-1 subtype B' accounts for 37% (6 of 16). Most of Dehong URFs were found to share the precise recombination breakpoints with those in CRF07/08_BC, suggesting the possibility that CRF07/08_BC may have arisen among URFs emerged in Dehong area, where the earliest epidemic in China began in 1989. Intriguingly, however, it is noted that all the enhancer-promoter sequences in LTR of Dehong URFs belong to HIV-1 subtype C that possesses the characteristic three NFκB motifs. In contrast, immediate downstream regions, encompassing from dimer initiation sequence to the amino-terminus of gag region, belongs to either subtype B' or C at equal frequency. The predominance of HIV-1 subtype C LTR in Dehong URFs suggests the biological advantage of LTR of subtype C over that of subtype B in in vivo recombination event. The HIV-1 subtype C LTR is known to show stronger transcriptional activity upon activation than LTR of other subtypes. The higher replicative potential of subtype C due to three NFκB configuration may explain the apparent selective advantage of subtype C LTR observed in the present study.

ASSOCIATION OF DC-SIGNR POLYMORPHISMS WITH HIV HIGHLY EXPOSED BUT SERONEGATIVE WIVES OF HIV-SEROPOSITIVE HUSBANDS IN NORTHERN THAILAND

Nuanjun Wichukchinda[1,3], Archawin Rojanawiwat[1], Yoshihiro Kitamura[2], Emi E. Nakayama[3], Panita Pathipvanich[4], Wattana Auwanit[1], Pathom Sawanpanyalert[1], Aikichi Iwamoto[2], Tatsuo Shioda[3], and Koya Ariyoshi[5]

[1]National Institute of Health of Thailand, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand; [2]Institute of Medical Science, University of Tokyo, Tokyo, Japan; [3]Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; [4]Day Care Center, Lampang Hospital, Lampang, Thailand; [5]AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan

To identify gene polymorphisms associated with HIV-exposed seronegative (ESN) individuals in Thais, genomic DNA from 297 HIV-seropositive and 93 HIV-seronegative spouses of HIV-seropositive partners were genotyped for candidate host gene polymorphisms (including the single-nucleotide-polymorphism (SNP) in Exon 5 of DC-SIGNR (IMS-JST025121G/A) and a variable-number-of-tandem-repeats polymorphism in Exon 4 of DC-SIGN-R). We found that two polymorphisms in DC-SIGNR (CD209L) were significantly associated with ESN females but not ESN males. The IMS-JST 025121A and 5-repeat of a 69-bp were found at more significant frequency in HIV-seronegative than HIV-seropositive females ($p=0.028$ and $p=0.007$, respectively). These observations suggest that DC-SIGNR and/or genes in linkage-disequilibrium with DC-SIGNR play an important role in the establishment of HIV-infection in heterosexual female population.

Acknowledgements: We are grateful to all the participants of the Lampang cohort and blood donors. We also thank Suthira Kasemsuk, Sriprai Sanitwong-na-ayuthaya, Anong-nard Suyasarojna, Nutira Boonna, Prapan Wongnamnong, and Wimala Inunchot for their technical assistance.

NEW APPROACHES TO STUDY FLAVIVIRUS ENTRY AND INHIBITION

Robert W. Doms

University of Pennsylvania School of Medicine, Philadelphia, PA

West Nile virus, a flavivirus, has spread throughout North America over the past 5 years. We have developed entirely DNA-launched systems that make it possible to rapidly manipulate WNV proteins, introducing them into both replication competent and reporter virus particles. Using these systems, we have validated a high-throughput virus neutralization assay that is not subject to the vagaries of the more standard plaque reduction neutralization titer assay. In addition, we have sought to identify WNV receptors, and have explored the role that viral glycosylation plays in tropism and pathogenesis. The C-type lectins DC-SIGN and DC-SIGNR bind mannose-rich glycans with high affinity. *In vitro*, cells expressing these "attachment factors" efficiently capture, and are infected by, a diverse array of appropriately glycosylated pathogens, including dengue virus. We found that DC-SIGNR promotes WNV infection much more efficiently than DC-SIGN, particularly when the virus was grown in human cell types. The presence of a single N-linked glycosylation site on either the prM or E glycoprotein of WNV was sufficient to allow DC-SIGNR-mediated infection, demonstrating that uncleaved prM protein present on a flavivirus virion can influence viral tropism under certain circumstances. Preferential utilization of DC-SIGNR was a specific property conferred by the WNV envelope glycoproteins. Chimeras between DC-SIGN and DC-SIGNR demonstrated that the ability of DC-SIGNR to promote WNV infection maps to its carbohydrate recognition domain. WNV virions and subviral particles bound to DCSIGNR with much greater affinity than DC-SIGN. We believe this is the first report of a pathogen interacting more efficiently with DC-SIGNR than DC-SIGN. Our results should lead to the discovery of new mechanisms by which these well-studied lectins discriminate among ligands, as well as revealing properties needed for efficient WNV infection.

MULTIPLE MECHANISMS OF ENTRY INHIBITOR RESISTANCE: A MODERN TALE OF CERBERUS OR HYDRA?

Christos J. Petropoulos[1], Wei Huang[1], Jonathan Toma[1], Signe Fransen[1], Sebastian Bonhoeffer[2], and Jeannette M. Whitcomb[1]

[1]Monogram Biosciences, South San Francisco, CA. [2]ETH Zurich, Zurich, Switzerland

Virus entry is a multi-step process involving several virus envelope proteins (gp120SU, gp41TM) and host cell receptors (CD4, CCR5, CXCR4). The cascade of protein-protein interactions and conformational changes that mediate virus entry provide a number of novel viral and host-cell targets that are functionally and mechanistically distinct from conventional enzymatic targets. Current drug candidates in clinical development include small molecule inhibitors and monoclonal antibodies that target virus attachment (CD4-gp120 binding), co-receptor engagement (CCR5- or CXCR4-gp120 binding), and membrane fusion (gp41). Recent in vitro and in vivo observations indicate that resistance to entry inhibitors can differ significantly from that of protease and reverse transcriptase inhibitors, and may emerge via alternative mechanisms depending on the specific molecular interaction that is targeted.

Viruses with reduced susceptibility to various attachment and fusion inhibitors display log-sigmoid inhibition curves that typically reach 100% inhibition at high drug concentrations, consistent with a competitive mechanism of inhibition and escape. Reductions in susceptibility to these agents are best described by increases in the $IC_{50.}$ In the absence of complete suppression of viral replication, resistance to competitive entry inhibitors can emerge rapidly, consistent with the pre-existence of resistant variants at low levels in the quasi-species (analogous to certain NRTI (M184I/V) or NNRTI (K103N) resistant variants).

In contrast, viruses that are selected for replication in the presence of inhibitors that antagonize envelope-co-receptor, or envelope-receptor interactions, often exhibit inhibition curves that plateau before reaching 100% inhibition. The inability to block 100% of virus replication at elevated drug concentrations is consistent with an allosteric mechanism of inhibition of these agents. Viruses that develop resistance to co-receptor antagonists likely acquire the ability to bind and utilize receptor- or co-receptor-inhibitor complexes. Consequently, resistance to allosteric co-receptor inhibitors is best described by the extent of incomplete inhibition observed at high drug concentrations (the percent inhibition at which the curves plateau). Recent in vitro and in vivo data suggest that variants with reduced susceptibility to allosteric entry inhibitors can pre-exist in the viral quasi-species and emerge rapidly upon drug pressure, or may emerge more slowly requiring the successive incremental accumulation of resistance and compensatory mutations in envelope.

CYSTEINE PROTEASES ARE ESSENTIAL HOST FACTORS FOR FILOVIRUS INFECTION

Kartik Chandran[1], Jin-Yi Yang[1], Nancy Sullivan[2], and James M. Cunningham[1]

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Ebola and Marburg viruses cause sporadic outbreaks of rapidly fatal hemorrhagic fever. We show that endosomal cysteine proteases are essential host factors for infection of Vero cells and primary human macrophages by these viruses. Cathepsin B is essential for infection by the Zaire virus, however Marburg>Reston>Sudan>Cote d'Ivoire viruses also utilize cathepsin L. Infection is closely correlated with direct cleavage of the filovirus envelope glycoprotein GP. Based on analysis of GP cleavage products, we propose that the function of cysteine proteases in filovirus infection is analogous to receptor binding in retrovirus infection and endosome acidification in influenza virus infection. Importantly, inhibitors of cathepsin B/L block filovirus infection and therefore have potential as anti-viral drugs.

STRUCTURAL STUDIES OF THE PRE- AND POST-FUSION CONFORMATIONS OF THE PARAMYXOVIRUS F PROTEIN

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The paramyxoviridae are enveloped viruses that include, among others, mumps virus, measles virus, Sendai virus, Newcastle disease virus (NDV), human respiratory syncytial virus (HRSV), parainfluenza virus 5 (SV5) and human parainfluenza viruses 1-4 (hPIV). Like other enveloped viruses, such as influenza and HIV, the paramyxoviruses require fusion of the viral and cellular membranes to enter the host cell. The fusion (F) protein catalyzes this membrane merger and entry step and it has been postulated that F undergoes complex refolding during this process. However, it has remained unclear to what extent the F pre- and post-fusion conformations differ and how these are linked to membrane fusion. We have determined the crystal structures of the SV5 and hPIV3 F proteins, in the pre- and post-fusion conformations, respectively. We have previously published the structure of the uncleaved, secreted hPIV3 F ectodomain, truncated before the transmembrane (TM) domain. Unexpectedly, we found that this structure contains a 6-helix bundle associated with the post-fusion conformation, suggesting that the F TM domain and/or the cytoplasmic tail are important for the folding to or stability of the pre-fusion, metastable state. More recently, we have determined the crystal structure of the SV5 F protein in its pre-fusion conformation, after stabilizing the metastable state by the addition of a C-terminal trimerization domain. The pre-fusion F structure reveals major conformational differences between the pre- and post-fusion states, involving transformations in secondary and tertiary structure. The positions and structural rearrangements of key parts of the fusion machinery clarify how a novel metastable protein fold and its conformational transition to a more stable state can trigger membrane fusion. The structural results have implications for understanding the folding/refolding transitions in other class I viral fusion proteins, such as HIV Env.

FURIN-CLEAVAGE POTENTIATES THE MEMBRANE FUSION-CONTROLLING INTERSUBUNIT DISULPHIDE-BOND ISOMERIZATION ACTIVITY OF LEUKEMIA VIRUS ENV

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The membrane fusion protein of murine leukemia virus is an Env protein trimer. The Env consists of a peripheral (SU) and a transmembrane (TM) subunit that are linked by an intersubunit disulphide-bond. The latter is coupled to an isomerization motif, CXXC, by which the virus controls its fusion reaction. Upon receptor binding the isomerase rearranges the intersubunit disulphide-bond into a disulphide-bond isomer within the motif. This facilitates SU dissociation and fusion activation in the TM subunit. We have investigated whether furin cleavage of the Env precursor will potentiate the isomerase to become triggered. To this end we accumulated the late form of the precursor, gp90, in the cell by incubation in the presence of a furin inhibiting peptide. The isomerase was then triggered to become active by solubilization in NP-40 under alkylation-free conditions. This treatment has earlier been shown to be a powerful inductor of isomerization. As a non-isomerized control a parallel sample was solubilized under alkylating conditions. The precursor was immunoprecipitated, gel isolated, deglycosylated and subjected to complete trypsin digestion and the peptides separated in SDS-tricine-PAGE under nonreducing conditions. This analysis revealed the size of a characteristic major disulphide linked peptide complex that differentiate the two isomers of the Cys 336(9)-Cys 563 disulphide-bond, i.e. the bond corresponding to the intersubunit disulphide-bond. The isomerase was found to be at least eight fold more resistant to triggering in the precursor than in the mature, cleaved form. This suggests that the isomerase becomes potentiated for triggering by a structural change in Env that is induced by furin cleavage in the cell.

IMMATURE DENDRITIC CELL-DERIVED EXOSOMES CAN MEDIATE HIV-1 TRANS INFECTION

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Immature dendritic cell subsets resident in peripheral mucosal tissues are presumably the first cells targeted by HIV-1. In vitro data have demonstrated that immature dendritic cells (DCs) can bind HIV-1 for extended periods of time, and that bound virus particles can be subsequently transmitted to either quiescent or activated CD4+ T cells to induce productive infection. But the pathway by which an endocytosed virus particle is transmitted from DCs to CD4+ T cells remains unclear. To determine the mechanism of transfer of endocytosed virus particles, DCs were exposed to single cycle of replication competent luciferase expressing HIV-vectors (HIV-luc). Initiation of infections at 37°C protected virus particles from trypsin digestion, indicative of virus particle endocytosis. Endocytosed virus particles localized within tetraspan protein (CD9 and CD63) positive multiple vesicular body (MVB)-like compartments. Surprisingly, as opposed to lysosomal degradation, a significant fraction (25 – 30%) of the endocytosed virus particles were released into the extra-cellular milieu, and were infectious for CD4+ T cells. These exocytosed HIV-1 particles in DC-supernatants were associated with vesicles. HIV-1 bearing vesicles in DC-supernatants also expressed HLA-DR1, CD1b, and the tetraspan proteins, CD9 and CD63, a proteomic profile that is suggestive of an MVB origin, and hence were termed exosomes. Finally, exosome-associated HIV-1 particles had a higher infectivity potential than cell-free virus particles. These findings suggest that HIV-1 trafficking through DCs can escape lysosomal degradation pathways and can result in productive infection of interacting CD4+ lymphocytes via exosome-associated virus particles.

GM3-MEDIATED ENGAGEMENT OF RAFT-ASSOCIATED CD4 MODULATES MEMBRANE INTERACTIONS AND INHIBITS HIV-1 FUSION

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Cellular ceramide, sphingolipids (GSLs) and their metabolites have been suggested to play a role in HIV-1 infection and pathogenesis (Bioscience Reports, 25, 2005). Our previous studies show that receptor bearing B16 mouse melanoma cells are resistant to gp120-gp41-mediated fusion and the fusion activity is restored by pre-treatment of the B16 targets with PPMP, a GSL biosynthesis inhibitor (J.Virol. 78: 7360-8, 2004). B16 cells express exceptionally high levels of GM3, a raft-associated ganglioside that interacts with CD4. Hence, we postulated that the block in fusion was due to immobilization and/or differential segregation of the CD4 in these cells. Here, we investigated GM3-mediated modulation of CD4 localization in the plasma membrane of B16 cells by utilizing a CD4 mutant (RA5). RA5, when expressed in permissive cells supports HIV-1 entry despite its preferential localization into non-raft fraction (Popik&Alce, JBC, 279:704-712, 2004). B16 cells expressing the RA5 mutant and the cognate coreceptors (CXCR4 or CCR5) readily fused with cells expressing the corresponding HIV-1 Envs. In contrast, B16 cells expressing the coreceptors and wild type CD4 (Wt-CD4) failed to support fusion as previously reported. Sucrose density gradient analysis showed that wt-CD4 was associated with raft fraction, whereas RA5 partitioned into non-raft fractions. Examination of lateral diffusion of lipids and receptors in B16 cells (by fluorescence recovery after photo bleaching (FRAP)) demonstrated that Wt-CD4 (but not coreceptors or membrane lipids) mobility was significantly restricted in B16 cells (1.87±2x10-11cm2/sec) when compared with GSL-deficient mutant GM95 cells (20.9±11.7x10-11cm2/sec). However, we did not observe any significant difference in the mobility of wt-CD4 and the RA5 expressed in B16 cells. These results support our hypothesis that restriction of fusion in B16 cells results from in situ immobilization of wt-CD4 by raft-associated GM3 resulting in interference with subsequent viral envelope-coreceptor interactions critical for membrane fusion.

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TRANSMISSION GENETICS OF POSITIVE-STRAND RNA VIRUSES AND THE CHOICE OF ANTIVIRAL TARGETS

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Positive-strand RNA viruses have well-known characteristics that reduce or eliminate the effectiveness of antiviral drugs. Specifically, the high error rates of RNA-dependent RNA polymerization and the exponential amplification of viral genomes ensure that, within any given population, genetic variation will arise that can lead to the selection of drug-resistant genomes.

One strategy to avoid the selection of drug-resistant viral variants is to ensure that the inevitable drug-resistant variants are not selected out of the complex population in which they arise. To this end, we sought to identify "dominant drug targets": protein or RNA targets that, when rendered defective by drug binding, poison all viral genomes in the same cell. For example, pleconaril, which targets picornaviral capsid proteins, binds to capsid precursors and inhibits their assembly and subsequent function. In mixedly infected cells, capsid precursors encoded by different genomes co-assemble to form mixed capsids. We have shown that pleconaril-sensitive poliovirus can inhibit the growth of pleconaril-resistant virus during co-infection. We have undertaken a genomic screen for dominant drug targets in the poliovirus genome and have identified three genomic regions in addition to the capsid-coding region that are especially promising. The relevance of these findings to drug targeting for other positive-strand viruses will be discussed.

One strategy to avoid the selection and outgrowth of drug-resistant viruses is to ensure that drug-resistant viruses, when they arise, will show reduced fitness. A promising group of drugs for treatment of RNA viruses are mutagens, such as ribavirin, that increase the replicative error rates over the threshold of "error catastrophe". We have shown that, for poliovirus, a viral variant resistant to ribavirin simply displays higher replicative fidelity. But does a high-fidelity viral variant show reduced fitness? During infections of mice that express the human poliovirus receptor, we could show that the high-fidelity poliovirus variant was greatly attenuated with respect to wild-type virus, and that this relative attenuation was exacerbated in the presence of defined, revertable selective pressures. Therefore, we conclude that the high-fidelity viral variants that will arise upon selection in the presence of RNA mutagens are likely to display reduced fitness due to a positive role for viral quasispecies during growth in complex hosts.

INHIBITION OF HIV-1 PLUS-STRAND INITIATION BY NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

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Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are highly specific and potent non-competitive allosteric inhibitors of human immunodeficiency virus-1 (HIV-1) reverse transcriptase (RT). NNRTIs inhibit reverse transcription in a substrate length-dependent manner in biochemical assays and in cell-based HIV-1 replication assays suggesting a random and stochastic mechanism of inhibition of replication by NNRTIs. Surprisingly, we observe that NNRTIs potently and specifically inhibit plus-strand initiation in vitro under conditions in which little or no inhibition of minus-strand DNA synthesis is observed. In assays that recapitulate the initiation of the plus-strand DNA synthesis, inhibition is observed with an RNA PPT primer but not with a DNA primer of corresponding sequence and with wild-type RT but not with NNRTI resistant enzymes. Structural elements that dictate sensitivity to NNRTIs are revealed using analogs of the plus-strand initiation substrate. The data presented here suggest that specific inhibition of plus-strand initiation may be an important mechanism by which NNRTIs block HIV replication.

RESISTANCE MECHANISMS OF HIV-1 REVERSE TRANSCRIPTASE MUTANTS K65R, M184V, AND K65R+M184V TO NRTIS

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The HIV-1 RT resistance mutations K65R and M184V occur in patients individually and in combination. We sought to describe the susceptibilities of these mutants to NRTIs and to investigate the underlying resistance mechanisms. Viruses carrying K65R have reduced susceptibility to most NRTIs, but retain full susceptibility to AZT. M184V mutants have reduced susceptibility to 3TC, FTC, ddI, and abacavir, but remain susceptible to tenofovir, AZT, and d4T. When M184V was added to K65R, the susceptibility to tenofovir, AZT and d4T were slightly increased compared to K65R alone, but were decreased for 3TC, FTC, ddI, and ABC. Virus susceptibility to NRTIs may be explained by the combined effects of two known enzymatic mechanisms of resistance: altered NRTI binding/incorporation and altered NRTI excision after incorporation. We measured NRTI binding/incorporation using steady state enzyme kinetics (K_i/K_m) and NRTI excision by ATP-mediated excision in the presence of ATP and dNTPs. K65R, M184V and K65R+M184V mutant RT showed decreased binding/incorporation of most NRTIs with the exception of M184V which showed unaltered binding/incorporation of TFV and AZT. For AZT, K65R and K65R+M184V RT showed decreased AZT excision in addition to decreased binding/incorporation associated with K65R. In contrast to previous work showing decreased AZT excision by M184V RT, we observed NRTI excision rates in the presence of physiological concentrations of dNTPs that were similar to WT, and were increased for d4T by this mutant. In conclusion, for most NRTIs, K65R, M184V, and K65R+M184V mutant RTs primarily disrupt NRTI binding/incorporation which correlates with their drug susceptibilities in tissue culture. In the case of AZT, however, decreased binding/incorporation by K65R and K65R+M184V was counteracted by decreased excision resulting in WT susceptibility. The effects of the M184V mutant on excision appear to depend on the experimental system and their contribution to drug susceptibility remain to be fully determined.

INHIBITION OF FLAVIVIRUS INFECTION THROUGH SUPPRESSION OF VIRAL TRANSLATION AND RNA SYNTHESIS

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The flaviviruses West Nile (WN), dengue (DEN), yellow fever (YF), Japanese encephalitis (JE), and tickborne encephalitis (TBE) viruses are emerging or reemerging pathogens, and are classified as Category A-C priority pathogens. No effective therapy is currently available for clinical treatment of flavivirus infections. Here we present three developments toward flavivirus drug discovery. (1) Using reverse genetic systems of flavivirus, we have developed a set of high-throughput screening (HTS) assays. A luciferase reporter was engineered into a self-replicating subgenomic replicon (containing a deletion of viral structural genes) and into a full-length viral genome to monitor viral replication. Potential inhibitors could be identified through suppression of luciferase signals upon compound incubation. (2) Using the HTS assays, we have identified a novel triaryl pyrazoline inhibitor of flavivirus. The compound exhibits a broad spectrum of anti-flavivirus activity (WN, DEN, YF, and Saint Louis encephalitis viruses), but the compound does not inhibit HIV and DNA virus. Mode-of-action analyses showed that the compound inhibits WN and DEN viruses through suppression of viral RNA synthesis. (3) Two antisense phosphorodiamidate-morpholino-oligomers (PMO) were demonstrated to potently inhibit WN virus. One PMO targets the 5'-end 20 nucleotides of the viral genome, while the other targets the 3'CSI (an RNA element involved in genome cyclization). In cell culture, the PMOs reduced viral titers by 5 to 6 logs at a 5-μM concentration without cytotoxicity. Mode-of-action analyses suggest that the 5'End and 3'CSI PMOs inhibit WN virus through specific suppression of viral translation and RNA synthesis, respectively. The 3'CSI PMO markedly inhibited a broad-spectrum of flaviviruses because it targets an RNA sequence that is conserved among mosquito-borne flaviviruses. In vivo studies showed that the PMOs could partially protect mice from WN disease. Overall, the results suggest that the triaryl pyrazoline and the PMO inhibitors could be developed for potential therapy of flavivirus infections.

FUNCTIONAL COMPLEMENTATION AND COASSEMBLY OF GAG POLYPROTEINS FROM HIV-1 AND HIV-2, TWO DISTINCT HUMAN IMMUNODEFICIENCY VIRUSES

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HIV-1 and HIV-2 dual infection occurs frequently in certain regions of the world. Furthermore, these two viruses target similar host cell populations. To identify potential interactions between these two human pathogens, we examined whether HIV-1 and HIV-2 Gag proteins can coassemble and functionally complement each other. We generated HIV-1- and HIV-2-based vectors with mutations in either the nucleocapsid (NC) domain or the PTAP motif of Gag; compared with wild-type vectors, these mutants had drastically decreased viral titers in one round of virus replication. Coexpression of the homologous NC and PTAP mutants allowed the coassembly and complementation of the two mutant proteins resulting in recovery of virus infectivity. Coexpression of the mutant HIV-1 and HIV-2 Gag could also generate infectious viruses; furthermore, heterologous complementation in certain combination had similar efficiency as the homologous complementation. Additionally, we used bimolecular fluorescence complementation analysis to directly demonstrate that HIV-1 and HIV-2 Gag can interact and coassemble. Taken together, our results indicate that HIV-1 and HIV-2 Gag polyproteins can coassemble and functionally complement each other during virus replication, which, to our knowledge, is the first demonstration of its kind. These studies have important implications for AIDS treatment and the evolution of primate lentiviruses.

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# equal contribution

THE CONTRIBUTION OF REPLICATION CAPACITY TO EVOLUTION OF HIV REVERSE TRANSCRIPTASE INHIBITOR RESISTANCE

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The contribution of HIV replicative capacity to the evolution of reverse transcriptase inhibitor (RTI) resistance has not been elucidated despite its potential implications for therapeutic strategies. In this study we utilized a competitive fitness assay to assess the relative fitness of thirteen drug-resistant HIV mutants in the presence and absence of inhibitor. Among these were 41L/210W/215Y (pathway 1) and 67N/70R/219Q (pathway 2) that confer high-level resistance to zidovudine (thymidine analogue mutations, TAMs), as well as 103N and 181C that confer high-level resistance to nevirapine. The concentrations of inhibitor used in these studies reflect the IC_{50} (10ng/ml of nevirapine), or a typical serum concentration observed for individuals receiving daily nevirapine (150ng/ml) or zidovudine (1.2 ug/ml) treatment. Our experiments reveal that in the absence of zidovudine the pathway 2 TAMs 67N/70R and 67N/70R/219Q are fitter than their 70R progenitor species, and the acquisition of 41L by the pathway 1 TAM 215Y substantially increases its fitness. In the presence of zidovudine, 215Y is more fit than 70R and 67N/70R, and the pathway 1 TAMs 41L/215Y and 41L/210W/215Y are the most-fit, consistent with their prevalence in clinical samples. In competitions between 103N and 181C without nevirapine, 103N is the fitter species, which is reversed in the presence of 10ng/ml of nevirapine. Moreover, the fitness advantage of 181C increased in the presence of 150ng/ml nevirapine. From these studies we conclude that viral replicative capacity contributes substantially to the evolutionary pattern of TAMs and that, as for protease inhibitor resistance, mutations act in primary (increasing resistance) and secondary (increasing fitness) capacities. We also surmise that drug resistance and fitness are competing forces underlying the emergence of nevirapine resistant species 103N and 181C, consistent with the resistance pattern observed in mothers and their infants treated with this inhibitor.

REPLICATION IN A BOX: NUCLEOTIDE IMPORT AND INITIATION OF HCV RNA SYNTHESIS

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Several crystal structures of the hepatitis C virus NS5B protein have been determined with metal ions, single-stranded RNA, nucleoside-triphosphates or inhibitors bound to the active site cleft. Essential structural features conserved in flaviviral polymerases protrude into the active site and control nucleotide import and mediate de-novo initiation. HCV polymerase retains a rigid architecture in the presence of ligands and large-scale conformational changes during the catalytic cycle as seen in DNA polymerases have not been observed. Viral RNA synthesis appears to occur in a tightly confined, rigid space, which in turn presents challenges for drug design through the likely occurrence of cross-resistance.

HIV RNA STRUCTURES SUPPORT SEVERAL DISTINCT MECHANISMS OF STRAND TRANSFER

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We previously analyzed the role of RNA structure in the strand transfer reactions associated with HIV replication and recombination. Pausing of the RT, such as at the base of a hairpin, during synthesis on the donor template, allows a close series of RNase H cuts. This produces a site where a homologous acceptor template can invade to initiate transfer. We observed that the DNA primer terminus extends out ahead of the initial DNA-RNA hybrid. The hybrid expands to eventually catch the DNA 3'-end completing the transfer well into or beyond the hairpin. Creating an invasion site is one role by which pausing of synthesis can promote transfer. If the homologous region between donor and acceptor is expanded to include another site upstream of the hairpin that favors invasion, the base of the hairpin can serve as a catalyst for transfer of the DNA terminus. Hybrids expanding from the upstream invasion region catch the paused primer terminus and complete transfer at the hairpin base. In this way the pause produced by a hairpin can influence two steps of transfer, i. the creation of an invasion site, and ii. the terminus transfer step.

Moreover, the RNA folding environment around a hairpin strongly influences the efficiency of transfer. Evidence will be presented that the RNA template region 3' of the PBS can substantially increase efficiency of minus strong stop transfer at the 5' end of TAR, compared to an RNA lacking that region. The additional RNA accomplishes this by altering dynamic changes in RNA structure during synthesis. Such dynamic changes direct the RT synthesis pause pattern and the RNase H cleavage pattern in TAR, in a way that suppresses dead end transfer intermediates and promotes intermediates of transfer. Overall, local structures and the general folding of the RNA contribute to the mechanisms that produce transfer.

X-RAY STRUCTURE FOR AN RNASE H INHIBITOR BOUND TO HIV-1 REVERSE TRANSCRIPTASE

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We have determined a 3.0 Å resolution X-ray crystal structure of HIV-1 reverse transcriptase (RT) complexed with DHBNH, an RNase H inhibitor (RNHI). HIV-1 RT uses two enzymatic activites, a polymerase and an RNase H, to convert the viral genomic single-stranded RNA into double-stranded DNA suitable for integration into the host genome [1]. RNase H is essential for virus replication; however, very few small molecule inhibitors targeting this function have been reported, and there are no crystal structures of HIV RT in a complex with an RNase H inhibitor. DHBNH is an N-acyl hydrazone derivative that inhibits RNase H with an IC_{50} of 0.5 μM but does not inhibit the RT polymerase (IC_{50}>20 μM). Despite this specificity, the inhibitor binds more than 40 Å away from the RNase H active site, at a novel binding site in the palm of the p66 subunit, between the primer grip and the polymerase active site. The inhibitor partially overlaps the non-nucleoside RT inhibitor (NNRTI) binding pocket. The inhibitor appears to interact with the conserved residues Asp186 and Trp229, as well as with Tyr188, Lys223, Asp224, Pro226, Phe227, and Leu228. Certain substitutions on DHBNH can enhance interactions in the NNRTI binding pocket, resulting in “dual inhibitors” that inhibit both the polymerase and RNase H activities of HIV-1 RT. Our results are consistent with the view that binding of DHBNH alters the trajectory of the nucleic acid substrate, affecting the RNase H activity. Knowledge gained from this study provides new opportunities for structure-based drug design.

[1] Coffin, J. M.; Hughes, S. H.; Varmus, H. E. *Retroviruses*; Cold Spring Harbor Laboratory Press: Plainview, NY, 1997.

DELAYED CHAIN TERMINATORS REVISITED: THE RETURN OF THE …RING

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Nucleoside reverse transcriptase inhibitors (NRTIs), such as AZT and d4T, lack the 3'-OH group on the deoxyribose ring, and terminate further primer elongation by HIV-1 RT once they are incorporated. Because the analogs remain at the end of the primer strand, HIV-1 RT variants that are proficient at ATP-dependent pyrophosphorylysis can remove the blocking group, freeing the primer for continued elongation. We previously described the inhibition of HIV-1 and HIV-1 RT by a class of NRTIs (N-MC dNTPs) that is conformationally locked in the North conformation. The conformationally locked analogs, which have normal 3' OH group, were able to block viral DNA synthesis after additional normal nucleotides were added to the primer (delayed chain termination). The block appears to be the result of steric interference between the modified ring of the analog and the thumb subdomain of HIV-1 RT. Since the analogs are not at the end of the primer, they are not efficiently removed by drug-resistant HIV-1 RT mutants efficient at ATP-dependent pyrophosphorylysis. However, the analogs do not appear to be efficiently phosphorylated by cellular kinases, which prefer the South conformation, and thus do not effectively interfere with HIV-1 replication in vivo. To circumvent this problem, NRTIs that were not conformationally locked were analyzed. These analogs retain the 3'-OH group and have an additional modification on the 4'-C (either a methyl or ethyl group). The presence of the 4'-group on the sugar favors the North conformation, but the analog is not locked into the North conformation, which may allow cellular kinases to phosphorylate these compounds.

The 4'-Me dTTP analog behaves as a delayed chain terminator; however, polymerization appears to terminate at a different positions than for N-MC dTTP. The delayed chain termination is seen with both an RNA and a DNA template. 4'-Et dTTP behaves like a conventional chain terminator (for example, AZTTP) in that continued polymerization is terminated immediately after the analog has been incorporated. The NRTI-resistant HIV-1 variant M184V appears to prevent the incorporation of 4'-Et dTTP, but remains sensitive to 4'-Me dTTP. Both analogs can be removed by HIV-1 RT variants proficient at ATP-dependent excision; however, the removal of the 4' analogs is less efficient than is the removal of AZTMP. Neither analog appears to be efficiently phosphorylated by cellular kinases. Because different cellular kinases activate the different deoynucleosides, we will test analogs in which the same sugars are linked to different bases and we are also planning to test other modified sugars in an attempt to find an analog that is an effective delayed chain terminator that is efficiently phosphorylated.

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RNASE H DOMAINS OBTAINED FROM TREATMENT-EXPERIENCED PATIENTS INCREASE RESISTANCE TO AZT

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We recently proposed that a balance between degradation of HIV-1 RNA by RNase H and nucleotide excision is an important determinant of NRTI resistance (PNAS USA 102:2093-2098, 2005). We observed that mutations in the RNase H domain conferred high-level resistance to AZT and d4T, supporting our model that mutations reducing the rate of RNA degradation will confer NRTI resistance by increasing the time period for excision of incorporated NRTIs from terminated primers. In the current studies, we sought to determine whether mutations in the RNase H domain that confer resistance to NRTIs are selected in response to therapy.

We performed genotypic analysis of the polymerase and RNase H domains derived from eight NRTI-experienced and seven naïve patients. The RNase H domains were cloned into HIV-1 vectors that contained a wild type polymerase domain or polymerase domain encoded TAMs and expressed a luciferase reporter gene. Susceptibility to AZT was determined during a single cycle of infection.

The RNase H domains obtained from seven drug-naïve patients did not increase resistance to AZT. In contrast, the RNase H domains obtained from six drug-experienced patients conferred increased resistance to AZT (IC_{50}, 0.12–0.29 μM; 2.4- to 5.7-fold) in the context of a wild type polymerase domain ($P < 0.05$). Remarkably, four of the eight RNase H domains obtained from treatment-experienced patients conferred substantially increased resistance to AZT (IC_{50}, 9.5–93.8 μM; 270- to 1840-fold) in the context of a polymerase domain encoding TAMs ($P < 0.05$).

These results indicate that RNase H domains that confer substantial resistance to AZT are selected in response to NRTI therapy. The selected RNase H domains may reduce the rate of RNA degradation and, in the context of TAMs, increase AZT resistance in a synergistic manner. These studies indicate the need to include the RNase H domain in future genotypic and phenotypic analyses of NRTI resistance.

NNRTI RESISTANCE MUTATIONS IN THE 51KDA SUBUNIT β7–β8-LOOP OF HIV-1 REVERSE TRANSCRIPTASE

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A yeast-based chimeric Ty1/HIV-1 RT (TyHRT) retrotransposon system detected several rare and novel NNRTI mutations in clinical samples, some of which occurred at residues 132 and 135 in HIV-1 RT. In order to understand the role of residues 132 and 135 as well as surrounding residues in NNRTI resistance, we constructed 12 recombinant HIV-1 RT enzymes harboring mutations at codons 132, 135, 136, 137, 138, 139 and 140. The effect of the mutations on HIV-1 RT structure, function, and drug resistance were analyzed in a subunit specific context. Many of the mutant enzymes exhibited significantly reduced DNA polymerase. Using the yeast two-hybrid assay for HIV-1 RT dimerization we show that the decrease in enzyme activity is probably due to the mutations disrupting the enzyme's inter-subunit interactions. Subunit selective mutagenesis revealed that the effect of these mutations on RT dimerization was mediated mainly through the p51 subunit. Drug resistance testing demonstrated that the mutations I132M, I135A and I135M generated high level resistance to nevirapine and delavirdine (>10-fold) and low level resistance to efavirenz. Mutations at codon 138 in RT also yielded cross-resistance to all NNRTI tested. Subunit selective mutagenesis again revealed that the effect of these mutations was mediated through the p51 subunit. None of the mutations gave resistance to nucleoside inhibitors. Taken together, our results highlight an important role for the β7–β8-loop in the p51 subunit of HIV-1 RT in drug resistance and protein stability.

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INTRODUCING UNNATURAL TYROSINE ANALOGS AT THE STERIC GATE OF HIV-1 RT CONFERS RESISTANCE TO 3TC

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To investigate how subtle chemical and structural changes in the amino acid side chain can affect nucleotide substrate selection in HIV-1 reverse transcriptase (RT), tyrosine analogs were substituted for Tyr115 of p66 RT. RT variants containing *meta*-Tyr, *nor*-Tyr, aminomethyl-Phe, and 1- and 2-naphthyl-Tyr were produced in a coupled *E. coli* transcription/translation system. Mutant p66 subunits were reconstituted with wild type (WT) p51 RT and purified to near homogeneity by affinity chromatography. Each modified enzyme retained DNA polymerase activity following this procedure. Aminomethyl-Phe115 RT incorporated dCTP more efficiently than WT and was resistant to 3TCTP when examined in a steady-state fidelity assay. 2-Naphthyl-Tyr115 RT inefficiently incorporated dCTP at low concentrations and was kinetically slower with all dCTP analogs tested. Models of RT containing these side chains reveal that aminomethyl-Phe115 substitution provides new hydrogen bonds through the minor groove to the incoming deoxynucleoside triphosphate and the template residue of the terminal base pair. These hydrogen bonds likely contribute to the increased efficiency of dCTP incorporation. In contrast, models of HIV-1 RT containing 2-naphthyl-Tyr115 reveal significant steric clashes with P157 of the p66 palm subdomain, necessitating rearrangement of the active site.

SUPPRESSION OF HIV-1 PROTEASE INHIBITOR RESISTANCE BY PHOSPHONATE-MEDIATED SOLVENT ANCHORING

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Cross-resistance among HIV protease inhibitors is a limiting factor in the broader clinical use of this class of antivirals. Here we report on the effective suppression of PI resistance using a covalent attachment of a phosphonate ester motif to a peptidomimetic inhibitor scaffold. Resulting inhibitors maintain high binding affinity to HIV-1 protease, potent antiviral activity, and unlike the parent molecule, display no loss of potency due to resistance mutations in the protease. Crystallographic analysis revealed full exposure of the phosphonate group to the solvent with no specific enzyme interactions. We term this effect solvent anchoring and demonstrate that it is driven by a favorable change in the inhibitor binding entropy that compensates for the enthalpy losses due to resistance mutations in the protease active site. This unique thermodynamic behavior is a result of increased degeneracy of the inhibitor binding states, allowing its effective molecular adaptation to the expanded cavity volume of the mutant proteases.

HTLV-1 AVOIDS APOBEC3G RESTRICTION BY A CIS-ACTING EXCLUSION MECHANISM MEDIATED BY THE C-TERMINUS OF NC

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APOBEC3G is a cellular cytidine deaminase that is packaged into HIV-1 virions and converts cytosines to uracils in minus strand DNA after reverse transcription. HIV-1 Vif counteracts the antiviral effects of human APOBEC3G (hA3G) by promoting hA3G degradation. Both HTLV-1 and HIV-1 infect T-cells *in vivo* and are likely to encounter hA3G during the course of infection. HTLV-1 is noted for its high replication fidelity and very low sequence diversity, both within individuals and within populations; but HTLV-1 does not encode a Vif protein. In single-cycle replication assays, HTLV-1 infectivity was less susceptible to hA3G inhibition than HIV-1. HTLV-1 virions and virus-like particles (VLPs) packaged hA3G much less efficiently than vif-minus HIV-1 VLPs. HTLV-1 does not encode a trans-acting protein to diminish virion incorporation of hA3G. Instead, a peptide motif in the carboxyl-terminus of NC acts *in cis* to exclude hA3G from HTLV-1 virions. Hence, HTLV-1 has evolved a fundamentally different way to avoid the antiviral effects of hA3G than HIV-1. Elucidating the mechanism for cis-acting exclusion of hA3G by HTLV-1 NC will illuminate how APOBEC3G is encapsidated.

A GAGGLE OF RETROVIRUS RESTRICTION FACTORS

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A number of different host restriction factors have been described. They include the murine Fv1 gene product that restricts MLV, Trim5alpha from primates that is capable of restricting a variety of viruses and the product from the Owl monkey Trim5 locus that restricts HIV. Despite significant efforts, little is known of the precise mechanism of restriction. In owl monkeys, the B30.2 domain of Trim5 has been replaced by cyclophilin A (CypA) through a pseudogene insertion. Restriction of HIV-1 by the resulting Trim5CypA fusion protein depends on CA binding to CypA, suggesting both that the B30.2 domain might be involved in CA binding and that the tripartite motif (RBCC) domain can function independently of the B30.2 domain in restriction. To investigate the potential of RBCCs from other Trims to participate in restricting HIV-1, CypA was fused to the RBCC of Trim1, Trim18 and Trim19 and tested for restriction. Despite low identity within the RBCC domain, all fusion proteins were found to restrict HIV-1 but not the non-binding mutant G89V, indicating that the overall structure of RBCC and not its primary sequence was important for the restriction function. The critical interaction between CA and Trim-CypA appears to take place soon after viral entry. Quantitative PCR analysis on viral reverse transcriptase products revealed that the different fusion proteins block HIV-1 at two distinct stages of its lifecycle. With Trim1 and Trim18, this timing is dependent on the length of the Trim component of the fusion protein. However, it does not appear dependent on restriction factor localization since Trim5, Trim5-CypA and both Trim1-CypAs show very similar cellular distribution patterns, including highly mobile cytoplasmic bodies associated with the microtubule network. These observations suggest that restriction factor binding can have different mechanistic consequences.

EVOLUTION OF HIV-1 GAG TO RESIST AN EARLY, POSTENTRY REPLICATION BLOCK

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A cDNA encoding a C-terminally truncated form of CPSF6 was identified in an expression screen for factors that interfere with HIV-1 replication. Infection by different primate lentiviruses, including replication competent X4-tropic and R5-tropic HIV-1, HIV-2, and SIV, is strongly inhibited by truncated CPSF6 whereas infection by the gammaretrovirus MLV is not. The infection block is postentry and appears to either interfere with the proper trafficking of the HIV-1 reverse transcription complex or stability of the viral cDNA genome. To understand the mechanism of interaction of truncated CPSF6 with HIV-1, we took two approaches. First, we sought to delineate regions of MLV that might accord resistance to truncated CPSF6. Use of single-round infectious HIV-1 vectors encoding portions of MLV Gag revealed that the MLV CA was necessary to confer resistance to truncated CPSF6. Second, we selected different HIV-1 isolates in $CD4^+$ T cells stably expressing truncated CPSF6. One culture infected with an R5-tropic HIV-1 eventually propagated infectious virus despite the presence of truncated CPSF6. Reinoculation of this virus in a new culture of $CD4^+$ T cells expressing truncated CPSF6 resulted in rapid, unimpaired virus production. Characterization of the resistant virus stock revealed several mutations including a change in HIV-1 Gag conserved in known HIV-1, HIV-2, and SIV isolates. Introduction of mutated Gag sequence in HIV-1 vectors conferred resistance to truncated CPSF6. In addition, FIV, which differs from primate lentiviruses in this region of Gag, was tested and found to be resistant to truncated CPSF6. These data highlight the role of Gag in determining retroviral susceptibility to early, antiviral factors. Examining the uncoating and trafficking of HIV-1 resistant to truncated CPSF6 will help elucidate the restriction mechanism and may identify cellular machinery intrinsic to primate lentiviral replication.

HIV-1 TAT PROTEIN TARGETS PROTEIN PHOSPHATASE-1 TO THE NUCLEUS TO REGULATE THE ACTIVITY OF CDK9

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Transcription of HIV-1 genes is activated by HIV-1 Tat protein that induces phosphorylation of the C-terminal domain of RNA polymerase-II by CDK9/cyclin T1. We previously showed that Tat-induced HIV-1 transcription is regulated by protein phosphatase-1 (PP1). In the present study we demonstrate that Tat interacts with PP1 and that disruption of this interaction prevents induction of HIV-1 transcription. We show that PP1 interacts with Tat in part through the binding of V36 and F38 of Tat to PP1 and that Tat is involved in the nuclear and subnuclear targeting of PP1. The PP1 binding mutant Tat-V36A/F38A displayed a decreased affinity for PP1 and was a poor activator of HIV-1 transcription. Surprisingly, Tat-Q35R mutant that had a higher affinity for PP1 was also a poor activator of HIV-1 transcription, because strong PP1 binding competed out binding of Tat to CDK9/cyclin T1. Inhibition of PP1 in vitro or in vivo prevented phosphorylation of RNAPII CTD indicating that PP1 might regulate the activity of a RNAPII CTD kinase. Stable expression of the central domain of NIPP1 (cdNIPP1) strongly inhibited HIV-1 transcription similar to the inhibition achieved with CDK9-directed siRNA. Expression of cdNIPP1 reduced association of Tat with PP1 but not with CDK9/cyclin T1. Also expression of cdNIPP1 prevented CDK9 phosphorylation; inhibited enzymatic activity of CDK9 in vivo; and reduces association of CDK9 with 7SK RNA. Inhibition of PP1 by cdNIPP1 did not have a pronounced effect on cellular transcription as determined by gene array and quantitative PCR analysis. Our study shows that nuclear PP1 controls phosphorylation and the activity of CDK9 in vivo and that HIV-1 Tat might target PP1 to the CDK9/cyclin T1 to upregulate the activity of CDK9. Our results also demonstrate as a proof-of-principle that selective inhibition of nuclear PP1 is a feasible approach to inhibit HIV-1 viral transcription.

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A TALE OF TWO ENDOSOMES: THE ROLE OF AP-3 AND VPU IN HIV PARTICLE ASSEMBLY

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HIV particles assemble on the plasma membrane of infected lymphocytes and epithelial cells. Intracellular assembly of virions is prominent in macrophages, and recent studies have revealed the multivesicular body (MVB) as the site of intracellular assembly. We recently reported a direct interaction of Gag with the AP-3 clathrin-like adaptor complex. Disruption of the Gag-AP-3 interaction prevented Gag from reaching the MVB and resulted in markedly decreased virion production. These studies suggest that Gag trafficking to the MVB is part of a productive assembly pathway. We next sought to determine the role of AP-3-mediated trafficking using cells from patients who are genetically-deficient in AP-3 complex function. Fibroblasts from patients with Hermansky-Pudlak syndrome Type II (HPS2) lack functional AP-3 complexes due to the production of a defective β subunit. HPS2 cells demonstrated deficient production of HIV particles, and Gag was found on small cytoplasmic vesicles that did not colocalize with CD63. Rescue of particle assembly was achieved through expression of an intact AP-3 β subunit in HPS2 cells.

Vpu is a viral accessory protein that enhances particle assembly in human cells. In this study, we asked whether endosomal compartments are essential to the enhancing effect of Vpu on HIV assembly. Vpu was found within the endoplasmic reticulum and trans-golgi network. In addition, a subfraction of Vpu was present on endosomal membranes. A substantial fraction of Vpu colocalized with markers of the pericentriolar recycling endosome (RE). To probe the importance of this compartment, we used dominant-negative approaches to block exit of molecules from the RE. Remarkably, interventions that blocked exit from the RE completely inhibited Vpu-mediated enhancement of particle release in human cells, while having no effect in permissive (AGM) cells. These studies suggest an essential role for the RE in the ability of Vpu to enhance HIV assembly/release.

PACKAGING OF CELLULAR MRNAS IN PSI⁻ HIV-1 AND MLV PARTICLES

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Selection of genomic RNA is an essential step in the formation of infectious retrovirus particles. As it is not well understood in molecular terms, it has not been exploited in the development of antiviral strategies. Particles are efficiently produced in the absence of packageable viral genomic RNA; these "psi⁻ particles" contain cellular mRNA molecules instead of viral RNA. To gain insight into the selection of RNA during particle assembly, we have compared the mRNA populations in psi⁻ HIV-1 and MLV particles with the mRNA populations in the virus-producing cells. Particles were produced following transient transfection of Gag-expressing plasmids into 293T cells. RNA was isolated from the particles and from the cells, and the RNAs were analyzed on Affymetrix microarrays.

Remarkably, the vast majority of the mRNAs in the particles are an unselected sample of cellular mRNAs. That is, their representation in virus is the same as their representation in the cell. One exception to this generalization is that mitochondrial mRNAs are quite abundant in total cellular mRNA, but are virtually absent from the virus particles. This finding is consistent with the fact that virus is assembled in the cytoplasm, where mitochondrial RNAs are unavailable for encapsidation. This exception is evidence that our "viral" preparations are not predominantly composed of cellular debris.

We also found that a small number of cellular mRNA species are significantly enriched in virus particles. The same species are enriched in both MLV and HIV-1 particles and, despite their enrichment, only constitute a minute fraction of the total RNA in these particles. It is striking that, while the normal packaging of viral RNA is highly selective, the packaging of cellular RNAs is almost completely unselective. The results suggest that viral RNA, although it is an mRNA, is very different from cellular mRNAs in some way.

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VIRAL RESISTANCE TO PA-457, A NOVEL INHIBITOR OF HIV-1 MATURATION: INSIGHTS INTO THE DRUG TARGET AND MECHANISM OF ACTION

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The identification of new anti-HIV-1 drugs targeting novel sites of action remains a high priority in this era of drug-resistant viruses. One such new drug is PA-457, which potently inhibits both wild-type HIV-1 and isolates resistant to current anti-retrovirals. PA-457 disrupts virus maturation, a process essential for virus infectivity. Virus maturation requires the sequential proteolytic cleavage of Gag to matrix (MA), capsid (CA), nuclocapsid (NC), and p6 domains, and spacer peptides SP1 and SP2. PA-457 disrupts the cleavage of CA-SP1 to CA (Li et al, PNAS 2003). To elucidate further PA-457's target and mechanism of action, we have selected for and characterized PA-457-resistant viral variants by serial passage at a suboptimal drug concentration. We have identified five amino acid changes that independently confer PA-457 resistance. Three substitutions are located at the extreme C-terminus of CA (H226Y, L231M and L231F) and two were identified at the 1st and 3rd residues of SP1 (A1V and A3V). Mutations H226Y, L231M, L231F and A1V had little effect on viral replication kinetics in the absence of drug or with either suboptimal or high drug concentrations. The A3V change greatly decreased viral fitness in the absence of drug and at a suboptimal drug concentration. Interestingly, A3V fitness increased at a high drug concentration. Electron microscopy revealed that in the absence of drug A3V-infected cells exhibited an accumulation of Gag at the plasma membrane and released immature virions. In the presence of a high drug concentration, A3V-infected cells produced virions with condensed cores. The replication defect imposed by A3V was reversed by a second-site change in CA (G225S). The A3V/G225S mutant was highly fit and fully drug resistant. These results help define PA-457's molecular target and provide insights into the potential for development of PA-457 resistance in vivo, a pertinent matter as PA-457 is currently in phase II clinical trials.

PA-457 INHIBITS MATURATION OF THE HIV-1 GAG PRECURSOR ASSEMBLED IN VITRO

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PA-457, also called DSB, has been shown to potently inhibit HIV replication in culture (1, 3). In contrast to inhibitors that act upon the viral proteinase, PA-457 appears to block only the final maturational cleavage of p25CA-p2 to p24CA (1, 3). However, attempts to replicate this effect in vitro using recombinant Gag have failed leading to the hypothesis that activity is dependent upon the assembly state of Gag (1). Using a synthesis/assembly system for chimeric HIV-1 Gag proteins (2) we have replicated the activity of PA-457 in vitro. Processing of assembled chimeric Gag can be inhibited by the addition of drug with only the final cleavage of p25 to p24 blocked. Consistent with our hypothesis and with earlier findings, inhibition appears specific to Gag assembled into an immature capsid-like structure, since synthetic Gag that remains unassembled processes normally. To further analyze the authenticity of the assay, PA-457 was tested in parallel with its inactive parental compound, betulinic acid. Betulinic acid had no effect upon p25 processing in this system. Analysis of a PA-457–resistant mutant, A1V (1), in this system pointed to more rapid cleavage as a possible mechanism for resistance. However, characterization of additional mutations at the cleavage site and in p2 suggests that resistance does not strictly correlate with the rate of cleavage. With the establishment of an in vitro assay for the detection of PA-457 activity, the way is open to a more detailed characterization of its mechanism of action.

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THREE-DIMENSIONAL VISUALIZATION OF RETROVIRUSES AND OTHER PLEIOMORPHIC VIRUSES

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Structural analysis by X-ray crystallography and image reconstruction of cryo-electron micrographs has been highly informative for viruses whose architectures are uniquely defined and which observe a high degree of symmetry (usually icosahedral). However, many viruses are pleiomorphic and structurally variable, and consequently are not amenable to these approaches. Similarly, the interactions of viruses with cellular components during cell entry and exit (budding) are generally not synchronized over a population and therefore cannot be crystallized nor coherently averaged: these systems must be investigated on an individual basis. A recently developed three-dimensional imaging technique called electron tomography [1] shows considerable promise to elucidate the structures of pleiomorphic viruses and the interactions of virtually any virus – regular or otherwise – with host cells in near-molecular detail. This talk will explain the principle of ET, consider its limitations – in particular, accessible resolution – and illustrate its potential (e.g. [2]).

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IDENTIFICATION OF SMALL MOLECULE INHIBITORS OF HIV ASSEMBLY AND MATURATION

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The protein/protein interactions driving HIV-1 assembly and maturation represent novel and attractive targets for therapeutic intervention. Homotypic CA interactions, in particular, are key to forming both immature and mature virions. We have screened a library of 10,000 druggable compounds using an in vitro kinetic assay for the assembly of recombinant CA into tubes reminiscent of mature viral cores. With this assay we have identified ~100 compounds that inhibit assembly in the low micro-molar range. The compounds identified in this screen were also tested for their ability to inhibit the replication of HIV-1 strains NL4-3 and BAL at compound concentrations ranging from 0 - 75 μM in a 5 day Jurkat cell based assay. Cell viability was monitored concurrently. Ten compounds with a favorable IC_{50} and therapeutic index were selected. These compounds were then tested in a six-day assay using 5.25.EGFP.Luc.M7 cells. Six compounds with low micromolar IC_{50}'s and therapeutic indices of 15 or better were identified. A subset of these compounds were tested against virus replication in PBMC's and were found to be effective inhibitors. Compounds targeting CA can act as either assembly or maturation inhibitors. To discriminate between these possibilities the compounds were tested in single cycle experiments using transient transfection of 293T cells. The culture media was assayed for p24 levels and viral titer with the expectation that maturation inhibitors would display low infectivity but unaltered p24 levels. Whereas compounds that inhibited assembly would result in decreased p24 levels with unaltered infectivity when normalized for the amount of virus produced. Using this analysis compounds which appeared to preferentially affect either assembly or maturation were identified. Initial Western blot analysis suggests that none of the compounds act to alter Gag poly-protein processing. NMR and biochemical studies are currently underway to determine the binding site for each of these compounds.

AMINO ACID PREFERENCES FOR SUBSTRATE BINDING SUB-SITES OF RETROVIRAL PROTEASES IN TYPE 1 CLEAVAGE SITES

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The HIV-1 protease (PR) has proved to be an excellent target for antiretroviral therapy of AIDS, and various PR inhibitors are now in clinical use. However, there is a rapid selection of viral variants that are resistant to inhibitors of PR. Many of the mutations occurring in these variants are introducing residues that can be found in the corresponding position of other retroviral PRs. Therefore understanding the specificity similarities and differences of these enzymes may help to design broad-spectrum inhibitors against HIV-1 PR.

We have compared the specificity of the PR of 11 retroviruses representing each of the seven genera using a series of oligopeptides with amino acid substitutions in the P4-P1 positions of a naturally occurring type 1 cleavage site (Val-Ser-Gln-Asn-Tyr↓Pro-Ile-Val-Gln; the arrow indicates the site of cleavage) in HIV-1. Molecular models for all studied PRs were built, and they were used to understand the specificity similarities and differences between PRs and for interpretation of the results. The PRs had very similar preferences for P1 residues. In one of the most critical sites, at P2, size complementarity appeared to be the main specificity-determining feature, while electrostatic contributions may play a pronounced role only in case of HIV proteases. More variations were observed in preferences for outer (P3, P4) positions. The specificity distinction of the proteases correlated well with the phylogenetic tree of retroviruses prepared solely based on the PR sequences. Furthermore, the specificity of selected PRs was also compared using a large set of peptides representing naturally occurring cleavage sites of the studied enzymes. Inhibition profiling of selected PRs was also performed and the results suggested that the specificity of the retroviral proteases is more conserved as compared to their sensitivity towards inhibitors.

TIGHT BINDING NNRTI AFFECT THE LATE STAGES OF HIV-1 REPLICATION

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Previous studies have demonstrated that nonnucleoside reverse transcriptase inhibitors (NNRTIs) can enhance HIV-1 reverse transcriptase (RT) dimerization. Since p66 is part of the Gag-Pol polyprotein precursor we investigated whether NNRTIs affect the late stages of virus replication. Cells (293T and HeLa) transfected with wild-type NL4.3 and treated with efavirenz (EFV) and TMC125 showed more efficient processing of Gag-Pol and p66 to p51 compared to untreated cells. Enhanced processing of Pr55gag to p24 was also observed. Increased Gag and Gag-Pol processing was more dramatic in cells transfected with a myristoylation (-) mutant of NL4.3 suggesting that enhanced processing occurs in the absence of plasma membrane localisation. In contrast, cells treated with zidovudine and NNRTIs that are either weak binders (nevirapine, NVP) or that do not enhance RT dimerization (delavirdine) displayed Gag and Gag-Pol processing patterns similar to untreated cells. Examination of viral particle production from EFV and TMC125 treated cells revealed a 50% decrease compared to untreated and NVP treated cells. Significantly, we observed no decrease in intracellular p24 levels, cellular protein synthesis and viral particle production in MoMLV transfected cells at the drug concentrations tested indicating that the effect was not due to drug toxicity. EFV failed to decrease viral particle release of an HIV-1 protease (PR) active site mutant and NL4.3 containing the K103N mutation, which confers EFV resistance, indicating that this effect is dependent on a functional PR and appears to be mediated by the drug binding to p66 in the context of Gag-Pol. These data suggest that drugs enhancing Gag-Pol interaction in the cell cytoplasm can induce premature activation of the HIV-1 PR and a subsequent increase in intracellular Gag and Gag-Pol processing. These data demonstrate a novel mechanism of inhibition of HIV-1 replication by EFV and TMC125 in addition to their inhibitory effect on RT activity.

POSTER ABSTRACTS

POSTER 1

CHANGES IN PREVALENCE AND PATTERNS OF DRUG RESISTANT MUTATIONS IN JAPAN—SUMMARY OF NINE YEARS NATIONWIDE HIV-1 DRUG RESISTANCE MONITORING STUDY (1996 TO 2004)

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Objective: Highly active antiretroviral therapy (HAART) was introduced in Japan in 1997 and has been accepted as a standard treatment for HIV-1 since that time. As has been widely reported, HAART has improved the prognosis of the infection significantly. However, cases of failure in treatment due to antiretroviral drug resistance are increasing and becoming a critical issue that must be overcome. Here we present a summary of our seven-year surveillance study of drug resistant HIV-1 in Japan.

Methods: Blood samples sent to the AIDS Research Center, NIID, for routine drug resistance genotyping from November 1996 to December 2004 were analyzed. Drug resistance testing was performed using in-house genotyping protocols. Briefly, HIV-1 RNA was extracted from patient serum, and protease and RT fragments were amplified and sequenced.

Results and Discussion: During the study period, 6396 seropositive samples were collected. The prevalence of NRTI resistance was 44% in 1996, and increased up to 54% in 1997, the year when HAART was introduced, and remained above 50% throughout the period. The prevalence of PI resistance jumped from 0% in 1996 to 41% in 1999. Subsequently, PI resistance frequency decreased in 2000 to 2002, and reciprocally the prevalence of NNRTI increased from 5% in 1999 to 18% in 2002. The data appears to reflect the availability and trends of anti-retroviral treatment in Japan. PI was approved in 1997, prescriptions increased thereafter, and the increase in PI resistance matched this progression. Subsequent changes in the prevalence of PI resistance and NNRTI resistance coincided with the availability of efavirenz, which was approved in 1999 and experienced increased use as a replacement for PIs in HAART. Our data indicates high prevalence of drug resistance cases in anti-retroviral exposed population, and alert increasing risks of newly infections by drug resistance viruses.

POSTER 2

DISTINCT PATTERNS OF SEQUENCE VARIATION IN HIV-1 *PRO* AND *POL* IN CHRONICALLY INFECTED ANTIRETROVIRAL-NAÏVE INDIVIDUALS

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Backgound: Rapid and error-prone replication of HIV-1 yields a genetically diverse virus population from which drug resistant mutants are selected. The emergence of RT and PI mutations during combination antiretroviral therapy remains unpredictable, but may be related to baseline sequence variation. To characterize the diversity of pre-therapy HIV-1 populations and to determine whether *pro* and *pol* vary independently over time, we compared sequence variation and phylogenetic relationships of *pro* and *pol* in chronically infected drug-naïve individuals.

Methods: Individual HIV-1 *pro-pol* sequences (PR and nt 1-1200 of RT) were obtained by single genome sequencing. A total of 950 single genome sequences from 14 drug naïve individuals infected with HIV-1 for at least 1.5 years were obtained from samples spanning a study period of 1-14 years. Sequences were aligned using Clustal W and subjected to phylogenetic analysis; recombination was evaluated using the 4 gamete model of Hudson.

Results: Sequence analysis revealed comparable levels of genetic diversity of *pro* and *pol* within individuals. However, a position-specific analysis revealed that nucleotide variation was not uniformly distributed in HIV-1 *pro* or *pol*. In *pro*, synonymous and nonsynonymous changes were concentrated in 4 regions (nt 34-79, 101-130, 175-240, 260-290); nonsynonymous changes largely localized to solvent-exposed regions. *Pol* had few clusters of nonsynonymous polymorphisms in fingers and palm domains, but highly polymorphic synonymous changes were present throughout. Longitudinal analyses (N=9 pt) did not identify consistent changes in *pro* or *pol* diversity over time. Phylogenetic analyses revealed relatively homogenous HIV-1 *pro* and *pol* sequences within individuals; bootstrap analysis did detect distinct *pro* and *pol* phylogenetic relationships (bootstrap values > 85%) in 6/9 individuals, suggesting independent sequence evolution of the two genes. Recombination analyses revealed evidence of frequent recombination with minimum recombination intervals as short as 7-17 nt.

Conclusions: In contrast to HIV-1 samples taken from patients early in infection, sequence diversity in *pro* and *pol* is comparable and stable over time in chronically infected individuals. Position-specific analysis reveals that *pro* diversity is localized to specific regions. The presence of frequent recombination provides a potent mechanism for independent *pro* and *pol* variation.

POSTER 3

DIFFERENTIAL EVOLUTION OF *PRO*, *POL*, AND *ENV* IN PATIENTS RECENTLY INFECTED WITH HIV-1

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Background: Genetic variation of HIV gives rise to immune escape and drug resistance and contributes to pathogenesis. Little is known, however, about the rates and extent of genetic variation of different genes in HIV. We therefore studied HIV diversity and divergence in recently infected patients followed for up to 5 years after infection.

Methods: Longitudinal plasma samples (N = 49) were obtained from 6 persons with acute/early HIV-1 (first sample obtained 0-9 months after infection). Individual viral sequences (≥ 22 per sample) of protease (*pro)*, reverse transcriptase (*pol*) and envelope (*env*) were obtained with single genome sequencing. The extent of genetic variation was measured by average pairwise distance (APD) for each gene. Rates of diversification (APD/month) were compared among genes for each patient. Genetic divergence (relative to the earliest sample after infection) was measured by the method of Achaz et al. (*Mol. Biol. Evol.* 2004).

Results: Longitudinal analyses revealed heterogeneity in diversification and divergence of HIV genes. Diversification rates of *pro* and *pol* were relatively constant, ranging from no change to slight increases (slopes of 0 to 0.03), whereas diversification of *env* showed no consistent pattern, increasing and decreasing (ranging from 0 to 4.5% diversity) at different times in the same patient. By contrast, divergence of *pro*, *pol* and *env* increased consistently, although at a higher rate for *env* (slopes of 0.14–0.43 %/month) than for *pro* and *pol* (0.01–0.04 %/month). Greater divergence of env was primarily due to insertions, deletions, and duplications in the V1V2 loop. Although similar rates of accumulation of synonymous mutations were seen in *pro* and *pol*, there were 3-fold more nonsynonymous mutations (per # sites) in *pro*, which mapped to CTL epitopes matching the HLA type of patients.

Conclusions: The rates and patterns of HIV-1 diversification and divergence within individuals vary by gene: *env>>pro>pol*. Diversity in *env* can drop dramatically, indicating strong genetic bottlenecks, whereas diversity in *pro* and *pol* increase steadily. These differences are likely driven by varying selective pressures rather than differences in the underlying mutation rate. The predominance of nonsynonymous changes in *pro* and *env* imply positive selection to escape CTL and antibody pressure, respectively. The rarer occurrence of nonsynonymous changes in *pol* suggests less immune pressure or purifying (negative) selection.

POSTER 4

DRUG RESISTANCE MUTATIONS DETECTED IN RT-SHIV-INFECTED MACAQUES TREATED WITH A SINGLE DOSE OF NON-NUCLEOSIDE RT INHIBITOR (NNRTI)

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Animal models to study the evolution of resistance to clinical antiretroviral drugs are limited by the lack of activity of non-nucleoside reverse transcriptase inhibitors (NNRTIs) against SIV reverse transcriptase (RT). Therefore, we developed a pigtail macaque SHIV model to examine NNRTI therapy and resistance. SIV_{mne027} was modified by replacement of the RT coding region with that of HIV-1 to yield RT-$SHIV_{027}$. RT-$SHIV_{027}$ has an optimal tRNA-Lys3 PBS and an envelope tropic for monocytes/macrophages as well as $CD4^+$ T cells. Unlike wildtype SIV_{027}, RT-$SHIV_{027}$ was susceptible to several NNRTIs in both human and macaque indicator cell lines. Classical HIV-1 NNRTI resistance mutations in RT, including K103N and Y181C, were identified in the virus after *in vitro* selection with NNRTIs. After *in vitro* characterization, we challenged six pigtail macaques intravenously with RT-$SHIV_{027}$ to evaluate its pathogenesis and to examine its sensitivity to NNRTIs *in vivo*. Plasma RT-$SHIV_{027}$ RNA was measured using a quantitative real-time RT-PCR assay. A single, oral dose of the NNRTIs nevirapine (NVP) or efavirenz (EFV) was administered to macaques after establishment of a plasma viral load set point. HPLC was used to measure the presence of drug in the plasma and allele-specific real-time PCR was used to detect common resistance mutations in RT of virus isolated from plasma. RT-$SHIV_{027}$ peak plasma viral RNA levels averaged 6×10^6 copies/ml and plasma viral RNA was still readily measurable (10^3-10^7 copies/ml) after one year post-challenge. Total $CD4^+$ T cell counts showed a decrease over the course of the infection in many animals. Within 3-7 days following single dose EFV, the viral load declined 4- to 8-fold in all animals. By contrast, after NVP treatment, the viral loads did not show significant decreases, which could be due to the rapid metabolism and/or excretion of the drug in the macaques. The frequency of RT mutations K103N and/or Y181C was detected as high as 30% of the plasma virus from NNRTI-treated macaques. Our results suggest that the pigtail macaque RT-$SHIV_{027}$ model will be suitable for studies using NNRTI-containing combination antiretroviral therapy to examine the selection, relative fitness and persistence of resistant viral variants.

POSTER 5

CO-RECEPTOR USAGE OF HIV-1 ISOLATES FROM PATIENTS UNDERGOING LONG-TERM ANTIRETROVIRAL THERAPY

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The lack of CD4+ T-cell decline in some patients showing virus replication despite being under antiretroviral therapy has been explained by the selection of less fit viruses carrying drug resistance mutations. However, a shift in virus tropism with predominant infection of cells other than CD4+ T lymphocytes could also account for this observation. To further characterize the co-receptor usage of HIV-1 isolates from untreated and antiretroviral (ARV)-experienced individuals, HIV-1 isolates were collected from 21 patients: 7 drug-naïve and 14 ARV-treated. Drug resistance genotypes, C2V3 sequences of HIV-1 envelope gene and in vitro co-receptor usage were analyzed. ARV-experienced patients showed an average of 5.5 drug resistance mutations at the reverse transcriptase (RT) gene, as well as 2 primary and 4 secondary mutations at the protease gene. Drug-naïve patients carried 2 secondary protease mutations in average while none except for one isolate harbored RT resistance mutations. The virus isolates from all 7 ARV-naïve individuals and 71% (10/14) of the ARV-experienced patients showed CCR5 tropism, while 29% (4/14) of the ARV-experienced patients harbored CXCR4-tropic viruses. There was no apparent association between specific drug resistance genotypes and co-receptor usage. Our results indicated that all the viruses from HIV-1 infected patients on prolonged ARV therapy still use the classical chemokine co-receptors CCR5 or CXCR4 to infect cells. Thus, prolonged ARV treatment does not seem to favor the selection of viruses with a different cell tropism, which might influence its pathogenicity.

POSTER 6

C-TERMINALLY TRUNCATED CPSF6 INDUCES AN EARLY, POSTENTRY BLOCK TO HIV-1 REPLICATION

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A C-terminally truncated form of Cleavage and Polyadenylation Specificity Factor 6 (CPSF6) was identified in an expression screen for factors that interfere with HIV-1 replication. Expression of CPSF6-short form (CPSF6-SF) restricts HIV-1 infection by two orders of magnitude, while ectopic expression of wild-type CPSF6 (WT-CPSF6) does not affect susceptibility to HIV-1 infection. CPSF6-SF inhibits infection by replication-competent X4-tropic and R5-tropic HIV-1, HIV-2, SIVmac/mne, but not infection by MLV. RNA interference experiments indicate that WT-CPSF6 is neither required for HIV-1 replication, nor for CPSF6-SF antiviral activity. In contrast, siRNA depletion of CPSF6-SF mRNA transcripts restores susceptibility to HIV-1 infection. The infection block is post-penetration and independent of the virus entry pathway. Although WT-CPSF6 associates with nuclear pre-mRNA, CPSF6-SF is predominantly cytoplasmic and does not interfere with HIV-1 gene expression or the production of infectious virions. While the early steps of reverse transcription are unaffected and completion of second-strand synthesis is modestly decreased, the accumulation of 2-LTR circles is markedly reduced in cells expressing CPSF6-SF. These data imply that CPSF6-SF expression interferes with the nuclear import of the HIV-1 genome, or that the terminal ends of the viral DNA genome are incompletely processed or destabilized in the presence of CPSF6-SF. CPSF6-SF may thus provide a tool to identify cellular factors intrinsic to the lentiviral RTC-to-PIC transition.

POSTER 7

EVALUATION OF THE MODE OF ACTION OF ANTI-HIV ENVELOPE DRUGS

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To mediate viral entry, the gp120 subunit of the HIV envelope (gp120/gp41) binds to CD4 and CXCR4 or CCR5, allowing gp41 to force hemifusion and fusion of viral and cellular membranes. We have simultaneously quantified some of these events using an assay based on the coculture of chronically infected cells with non-stimulated primary CD4 T cells. In these cocultures, chronically infected cells transferred HIV particles to CD4 T cells, inducing the death and fusing with target cells. These events were evaluated by flow cytometry after staining p24 gag HIV antigen. Gag staining served to assess cell-to-cell transfer of HIV, while changes in cell morphology served to evaluate the death of single target cells. The disappearance of single CD4 T cells was used to assess the extent of cell-to-cell fusion. Analysis of the sensitivity of these parameters to drugs acting at different steps of HIV envelope function revealed characteristic inhibitory profiles of each group of drugs. Drugs targeting the binding of gp120 to CD4 completely blocked cell-to-cell transfer of HIV, single cell death and cell-to-cell fusion. Coreceptor or gp41 inhibitors acting after CD4 engagement blocked cell death and cell-to-cell fusion but increased cell-to-cell transfer of HIV particles. We provide a rapid method to evaluate gp120 binding to CD4, coreceptor-dependent and gp41 mediated hemifusion and cell-to-cell fusion. Our results also suggest that cell to cell HIV transfer could be used by HIV as an escape mechanism against HIV entry inhibitors.

POSTER 8

ALPHA-V INTEGRINS INTERVENE IN HIV-1 INFECTION IN MACROPHAGES

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Macrophages are key cells for HIV infection and spreading inside the organism. Macrophages cultured *in vitro* can be successfully infected after differentiation with cytokines like macrophage colony stimulating factor (M-CSF). In the monocyte to macrophage differentiation process with M-CSF, an upregulation of αv-integrins is produced concomitantly to the HIV capacity to replicate in monocyte derived macrophages. In the present study we show that an anti-αv antibody, 17E6, inhibited HIV-1 infection of primary macrophages. The effect of 17E6 on HIV-1_{BaL} replication in acutely-infected macrophages was dose-dependent, with a 50% effective concentration (EC_{50}) of 17 ± 2 μg/ml and without cytotoxic effects. Similarly, an anti-αvβ6 antibody, 14D9.F8, inhibited HIV-1_{BaL} replication in monocyte derived macrophages. 17E6 was able to reduce synthesis of HIV-1_{BaL} proviral DNA in acutely-infected macrophages after 18 hours post-infection, but was completely ineffective against HIV-1_{BaL} production in chronically infected macrophages, suggesting that this antibody inhibited HIV infection at an early stage of the virus cycle. Consistently, preincubation of differentiating monocytes with 17E6 decreased cell surface density of CD4, CCR5 and CD14 about 20, 40 and 50% respectively. Our results provide evidence for a role of αv-containing integrins in HIV replication in macrophages, and suggest that small molecular weight compounds may be developed to interfere with HIV replication in macrophages through interaction with αv integrins.

POSTER 9

ACTIVATION OF THE FUSION CONTROLLING INTERSUBUNIT DISULPHIDE-BOND ISOMERASE IN MLV ENV

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The SU and TM subunits of the murine leukemia virus (MLV) envelope protein complex (Env) are disulphide linked. This arrangement should prevent premature TM activation through SU dissociation, but also compromises receptor-mediated triggering of virus fusion at the plasma membrane (PM). To solve that problem MLV has evolved to include a Cys-X-X-Cys(CXXC)-linked disulphide-bond isomerase in SU. One of the Cys residues participates in the SU-TM disulphide-bond and the other carries a free thiol. This can be activated to attack the intersubunit disulphide-bond and cause its rearrangement into an internal disulphide-bond within the CXXC motif. We have studied the activation mechanism of the isomerase and the fusion reaction pathway that it controls. Using receptor positive and negative cells we showed that receptor binding triggers the isomerization reaction in Env. About 30% of the Envs isomerize in virus bound to rat XC-cells. The activation of the isomerase appears to involve the stabilization of the deprotonized, S-, form of the CXXC thiol by a conserved N-terminal His residue (His8). This was suggested by pH-titrations of the isomerization and fusion reactions and infection with MLV vectors that carry wt, His8 deleted or His8 substituted Env. The fusion activation pathway of Env was studied using a fusion-arrest assay. According to this the isomerization was blocked by alkylation of the CXXC thiol. This arrests Env in an intermediate, fusion-inactive, form that can be released by DTT reduction of the intersubunit disulphide-bond. We showed that isomerization precedes hemifusion of the viral and cell bilayers and that activation involves the conversion of TM from a prehairpin to a hairpin structure. Kinetic analyses of the alkylation-induced inhibition suggested that activation-induction of Env precedes the formation of presumed multi-Env fusion sites.

POSTER 10

RHESUS TRIM5-ALPHA IMPOSES AN EARLY REVERSE TRANSCRIPTION BLOCK ON CELL MEMBRANE FUSION OR ENDOCYTOTIC ROUTES OF HIV-1 INFECTION

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While CA appears to be associated with the reverse transcription complex (RTC) of the gammaretrovirus MLV, it is hypothesized that the CA shell of HIV-1 uncoats soon after virion penetration, and this step is necessary for the efficient initiation of reverse transcription. Biochemically purified HIV-1 RTCs capable of cDNA synthesis have little or no CA. In addition, CA mutations presumed to slow the rate of disassembly of the virion interfere with the initiation of reverse transcription. These data, however, do not a priori exclude an association of CA with the HIV-1 RTC in vivo. HIV-1 with mutations in CA thought to accelerate the disassembly of the virion core can also be impaired in the initiation of reverse transcription, and fluorescent imaging of intracellular HIV-1 shows that a majority of RTCs are associated with quantities of CA similar to the amount found in extracellular particles. The cytoplasmic body component TRIM5-alpha was recently identified as a factor that restricts the replication of both gammaretroviruses (Ref1) and primate lentiviruses (Lv1). A genetic interaction between retroviral CA and Ref1/Lv1 has been established. Primate TRIM5-alpha (Lv1) action depends on the presence of processed CA in an ordered, polymeric virion core. TRIM5-alpha thus provides a tool to examine the interaction of HIV-1 CA with the RTC. Recent studies of rhesus macaque TRIM5-alpha describe an early replication block to HIV-1 which, with VSV-G pseudotyped virus, appears to cause a reduction in the synthesis of minus sense strong stop DNA. Because VSV-G mediated entry of virus occurs via an endocytic pathway, the susceptibility of an intracellular virion core to cytoplasmic TRIM5-alpha could differ if virions enter cells via direct fusion to the cell membrane. The recently described Lv2 restriction of HIV-1 varies depending on entry pathway. Rhesus macaque cells endogenously expressing TRIM5-alpha or human cells that ectopically expressed rhesus TRIM5-alpha were challenged with HIV-1 via different pathways to compare the restriction by monitoring key steps in the synthesis of viral cDNA. Our data indicate that synthesis of minus sense strong stop DNA is strongly inhibited irrespective of HIV-1 entry pathway in the presence of rhesus TRIM5-alpha. We also found that rhesus TRIM5-alpha had modest effects on the later steps of viral cDNA synthesis in both human and rhesus cells. Reduced intracellular concentrations of rhesus TRIM5-alpha affected the efficiency of HIV-1 restriction but did not influence the stage of the block. These results suggest that soon after initiation of viral cDNA synthesis CA may be weakly associated with HIV-1 RTCs or is in a configuration not available to rhesus TRIM5-alpha.

POSTER 11

A STRUCTURAL MODEL OF CCR5 FOR THE DISCOVERY OF POTENT INHIBITORS FOR THE THERAPEUTIC INTERVENTION OF HIV-1 INFECTION

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CCR5 is an attractive target for therapeutic intervention of HIV-1 infection. Inhibitors of CCR5 are expected to have a very different resistance profile from reverse transcriptase or protease inhibitors. Currently several small molecule inhibitors of CCR5 are either in clinical or pre-clinical development. We have combined computational and experimental techniques in elucidating structures of CCR5-inhibitor complexes. After building an initial model using the structure of bovine rhodopsin as a template, the structure was iteratively improved by combing structure refinement and docking techniques. Experimental saturation binding assays guided the selection of the final model. This has enabled us to not only precisely define the binding site of CCR5 but also elucidate the key binding site interactions responsible for the anti-viral activity of CCR5 inhibitors. The model explains the relative affinity of AK602, currently in phase III clinical trial, and TAK-779 and SCH-C, which are potent inhibitors of HIV-1 infection. The model suggests important ideas for carrying out virtual screening to discover new novel inhibitors of CCR5.

POSTER 12

HIV-1 INTEGRATION REQUIRES THE VIRAL DNA END TO INTERACT WITH THE INTEGRASE FLEXIBLE LOOP

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Integration is essential for retroviral replication and gene therapy using retroviral vectors. HIV-1 integrase specifically recognizes the terminal sequences of the long terminal repeats (LTR) and cleaves their 3'-end terminal dinucleotides 5'-GT. The exposed 3'-hydroxyl is then positioned for nucleophilic attack and subsequent strand transfer into another DNA duplex (acceptor or chromosomal DNA). We report that both the terminal cytosine at the protruding 5'-end of the LTR (5'-C) and the integrase residue Q148 are critical for strand transfer. We propose that strand transfer requires a conformational change of the integrase-viral (donor) DNA complex with formation of an H-bond between the N3 of the 5'-C and the amine group of Q148. Proximity of the 5'-C and Q148 was demonstrated by disulfide crosslinking. Crosslinking is inhibited by the inhibitor 5CITEP (and a dinucleotide but not by an inactive diketo acid or L-chicoric acid). This crosslinking assay may be used for mapping of inhibitor binding sites. These findings have implications for the molecular mechanisms coupling 3'-processing and strand transfer as well as for the molecular pharmacology of integrase inhibitors.

POSTER 13

HIGH FREQUENCY OF GENETIC RECOMBINATION IS A COMMON FEATURE IN PRIMATE LENTIVIRUS REPLICATION

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Recent studies indicate that HIV-1 recombines at exceedingly high rates, approximately one order of magnitude more frequent than that of simple gammaretroviruses such as murine leukemia virus and spleen necrosis virus. Currently, we do not know the mechanistic differences that caused the disparity of recombination rates in these viruses. We hypothesize that high rate of genetic recombination is a common feature in primate lentiviruses but not gammaretroviruses. Alternatively, it is also possible that HIV-1 is unique among primate lentivirusese in possessing high recombination rates. We cannot distinguish these two possibilities because HIV-1 is currently the only primate lentivirus with measured recombination rate.

To test our hypothesis, we established a flow-cytometry-based recombination system to measure the recombination rates of two other primate lentiviruses, HIV-2 and SIVagm, in one round of viral replication. We determined that, for markers separated by 588, 288, and 90 bp, HIV-2 recombined at rates of 7.34%, 5.48%, and 2.34%, respectively, whereas SIVagm recombined at rates of 7.78%, 5.61%, and 2.73%, respectively. The recombination rates from HIV-2 and SIVagm are comparable to the previously measured HIV-1 recombination rates. Taken together, our results indicated that HIV-1, HIV-2, and SIVagm all recombine at very high frequencies, and the high recombination potential is most likely a common feature during primate lentivirus replication.

POSTER 14

HOW THE ROUS SARCOMA VIRUS REVERSE TRANSCRIPTASE HANDLES ALTERNATE POLYPURINE TRACTS

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During reverse transcription, the degradation of the RNA genome by the reverse transcriptase (RT) is generally nonspecific. However, RNase H must specifically cleave the tRNA primer used to prime minus-strand DNA synthesis, and specifically generate and remove the polypurine tract (PPT) primer for plus-strand DNA synthesis. The cleavage of the tRNA defines the right end of the linear viral DNA, and the cleavage and removal of the PPT defines the left end. Cleavage of the PPT is affected by a number of factors, including the sequence of the PPT. The PPTs of most retroviruses are similar, however, some retroviral RTs specifically recognize their cognate PPTs. In these studies, we substituted the endogenous PPT from RSVP(A)Z, Rous Sarcoma Virus (RSV)-based shuttle vector, and replaced it with alternate PPTs from different retroviruses and the Duck Hepatitis B virus “PPT-like” sequence. Substituting the endogenous RSV PPT with alternate PPTs reduced the relative titer by at least half compared to the wild-type virus. 2-LTR circle junction analysis showed that substituting alternate PPTs caused significant decreases of the fraction of viral DNAs with normal ends (consensus sequences), and significant increases in the insertion of part or all of the PPT. The last two nucleotides in the 3’ end of the RSV PPT are GA. Examination of the (mis)cleavages of the alternate PPTs revealed preferential miscleavages after “GA” dinucleotide sequences in a number of cases. When the terminal 3’ adenine of the RSV PPT was substituted with guanine, generating a PPT sequence similar to what is present in the MLV and HIV-1 PPTs, there was a preferential miscleavage after a GA sequence spanning the PPT/U3 boundary resulting in the deletion of the terminal adenine at the 5’ end of the U3. The relative titer of this virus was decreased compared to wild-type. An analogous substitution of the 3’ terminal guanine with adenine in the MLV PPT increased both the relative titer of the chimeric RSV-based virus and the fraction of consensus sequences detected in the 2-LTR circle junctions approximately 2-fold. Furthermore, the substitution eliminated the preferential miscleavage of the terminal adenine residue at the 5’ end of the U3. Additional studies of a chimeric virus containing the Duck Hepatitis B virus PPT-like sequence in reverse orientation revealed further insights into how RSV RT specifically cleaves the PPT/U3 junction.

POSTER 15

DETERMINING THE RATE LIMITING STEP OF HIV-1 REVERSE TRANSCRIPTASE STRAND TRANSFER

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Template switching during reverse transcription contributes to genetic recombination in HIV-1 when two non-homologous strains are co-packaged within the virus. Our recent studies suggest that strand transfer can occur through a multi-step mechanism on RNA templates involving RNase H cleavage, acceptor invasion, branch migration, and finally primer terminus transfer. Several labs have shown both a delay in the formation of transfer products relative to full length extension on the donor, and an increase in transfer efficiency with time, suggesting that transfers occur at a substantially slower rate than extension on the donor template. To better understand the steps involved in strand transfer this study has set out to define factors that alter the rate of formation of transfer products. Interestingly, increasing the acceptor concentration, increased the transfer efficiency but had no effect on the rate of transfer. This suggests that acceptor invasion is not the rate limiting step. Templates with a short region of homology preventing hybrid propagation also had a slow accumulation of transfer products. This suggests that hybrid propagation is not the rate limiting step. Substituting a DNA acceptor template and adding Klenow fragment to the reactions should increase re-initiation and extension exclusively on the DNA acceptor. This only leads to a small increase in the rate of transfer suggesting that the ability of the RT to re-initiate synthesize on the acceptor template is not the rate limiting step. These data suggest that the creation of the invasion site is likely to be the rate limiting step. Experiments are currently being performed to test this possibility.

POSTER 16

INSIGHTS INTO THE MULTIPLE ROLES OF PAUSING IN HIV-1 RT PROMOTED STRAND TRANSFERS

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We previously analyzed the role of hairpin structures within RNA templates in facilitating strand transfer by HIV-1 reverse transcriptase (RT). During synthesis RT stalls at the hairpin base, making a series of RNase H cuts in this region of the first RNA template (donor). A second homologous RNA template (acceptor) can then invade the donor-cDNA hybrid, interacting with the exposed regions of the cDNA and initiating the transfer. The acceptor-cDNA hybrid propagates by branch migration and eventually catches up with the extending primer terminus, later on the substrate. In this template system, where homology before the hairpin base was limited to 19nt, only 7% of transfers occurred at the pause-site, while 85% occurred within the hairpin. Interestingly, when the homology before the hairpin base was increased to 52nt, transfer efficiency increased to 3 fold. We also observed a noticeable shift in transfer distribution, with 32% of transfers now occurring at the hairpin base. Donor and acceptor cleavage profiles indicated that cDNA-acceptor interactions in this system were initiated well before the hairpin base, within the extended region of homology. The new results suggest that pause sites can serve as termination points for transfer. Stalling of synthesis at a pause site allows acceptor invasions initiated earlier in the template to catch up with the primer terminus. Details on the influence of template features and nucleocapsid protein (NC) on strand transfer will be presented.

POSTER 17

EFFECT OF *GAG* MUTATIONS ON RECOMBINATION AND RNA PACKAGING IN HIV-1

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HIV-1 recombination occurs during reverse transcription when parts of the two co-packaged RNAs are used as templates for DNA synthesis. It was hypothesized that HIV-1 packages RNAs *in cis*, that is Gag polyproteins preferentially encapsidate the RNA from which they were translated. This hypothesis implies that packaging-deficient *gag* mutants are selected against in two manners: these mutants do not generate infectious virus, and additionally, they are less likely to be rescued by wild-type viruses. Therefore, genetic information encoded by *gag* mutants will be rapidly lost in the viral population.

To test the *cis*-packaging hypothesis, we examined whether the RNA from packaging deficient *gag* mutants can be efficiently packaged by the proteins from the co-expressed wildtype virus. We further determined the recombination rates between *gag* mutants and wildtype virus. We used several packaging-deficient mutants including an NC zinc finger mutant, a CA truncation mutant, and a gag AUG mutant. We found that the viral RNAs from the NC or the CA mutant were packaged efficiently into virions when coexpressed with a wildtype virus, and these mutant viruses also recombined with wildtype virus at a rate similar to that of two wildtype viruses. In contrast, viral RNAs from mutants containing a 6-nt substitution encompassing the *gag* AUG were not efficiently packaged into virions, thereby causing a 20-fold decrease in the recombination rate between the mutant and wildtype viruses. Further analyses revealed that other more subtle mutations changing the *gag* AUG and abolishing Gag translation did not interfere with efficient encapsidation of the mutant RNA. These results indicate that neither the *gag* AUG sequence nor Gag translation is essential for viral RNA encapsidation. Taken together, our results indicate that *gag* mutant RNA could be efficiently packaged by wild type Gag polyprotein. Therefore, we propose that HIV-1 RNA encapsidation occurs mainly *in trans*.

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POSTER 18

ROLE OF CDK2 IN HIV-1 TRANSCRIPTION AND VIRAL REPLICATION

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Transcription of HIV-1 genes is activated by HIV-1 Tat protein, which induces phosphorylation of RNA polymerase II (RNAPII) C-terminal domain (CTD) by CDK9/cyclin T1. Earlier we showed that CDK2/cyclin E phosphorylates HIV-1 Tat and induces HIV-1 transcription in vitro. Here we show that Tat is a substrate for CDK2 in vivo and that RNA interference directed to CDK2 inhibits phosphorylation of Tat and also HIV-1 transcription and viral replication. Flag-tagged Tat expressed in HeLa cells was found to be phosphorylated in the cells metabolically labeled with ^{32}P. Inhibition of CDK2 expression by RNA interference greatly reduced phosphorylation of Tat, indicating that CDK2 may phosphorylate Tat in vivo. Tat was phosphorylated exclusively on serine residues in vivo, and studies in vitro identified the highly conserved Ser16 and Ser46 residues of Tat as the sites of phosphorylation by CDK2/cyclin E. Mutations of these residues inhibited Tat-induced HIV-1 transcription and prevented induction of HIV-1 viral replication. Inhibition of CDK2 expression by RNA interference inhibited Tat-induced HIV-1 transcription but not basal HIV-1 transcription or transcription from CMV or β-actin promoters. Inhibition of CDK2 potently blocked tumor necrosis factor alpha-induced HIV-1 viral replication in OM10.1 cells. Our results indicate for the first time that Tat is phosphorylated in vivo by CDK2 and suggest that phosphorylation of Tat might have a regulatory role in HIV-1 transcription. Our results also indicate that CDK2 participates in Tat-mediated HIV-1 transcription and may serve as a potential therapeutic target.

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POSTER 19

ISOLATION AND CHARACTERIZATION OF HIV-1 RESISTANT TO THE ALBUMIN-CONJUGATED PEPTIDE-BASED FUSION INHIBITOR FB006M

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FB006M contains a chemical modification that allows it to form a 1:1 conjugate with human serum albumin (FB006M-HSA) to extend its in vivo half-life. FB006M-HSA is a potent inhibitor of laboratory and clinical isolates of HIV-1 in a variety of assay systems (PBMCs, MDM, MAGI, CEM-SS, etc.), with IC_{50} values in the low nanomolar range. Resistant virus was generated through serial passages in the presence of escalating doses of FB006M-HSA. Nine passages were completed, resulting in a final FB006M-HSA passage concentration of 6.4 μM. Subsequent sensitivity testing revealed approximately 50- to 160-fold resistance in the passage-nine virus. Cross resistance to T-20 was observed when evaluating against FB006M-HSA virus passages 6-9 (IC_{50} values ranging from 1.57 μM to >10 μM compared to <0.032 μM against wild-type HIV-1NL4-3). In contrast, a number of T-20 resistant viruses did not exhibit cross resistance to FB006M-HSA. Sequencing of the gp41 domain of the viral envelope gene was performed for the nine virus passages. All passages exhibited an Aspartic Acid to Glycine change at gp41 amino acid (AA) 36, an Asparagine to Histidine change at AA 114, a Methionine to Threonine change at AA 115, and an Isoleucine to Valine change at AA 193. In addition, passages six through nine contained a clear Glutamine to Lysine change at AA 40, while passage five had a mix of both Glutamine and Lysine at this location, suggesting a mixed population of virus. Similarly, passages seven through nine contained a clear Asparagine to Lysine change at AA 126, while passage six had a mix of both Asparagine and Lysine at this location, also suggesting a mixed population. Finally, the passage-nine virus contained an additional Lysine to Isoleucine change at AA 144. The relevance of specific mutations to resistance will be discussed.

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POSTER 20

PROTEIN FOOTPRINTING OF HIV-RT BY DIFFUSIBLE CHEMICAL PROTEASES

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A key step in the life cycle of HIV is the process of reverse transcription which is performed by the enzyme reverse transcriptase (HIV-RT). This enzyme consists of two subunits, p66 and its proteolytically processed form p51. Functionally, HIV-RT has DNA polymerase and RNaseH activities. Both of these activities are sites for potential inhibition. To this end, inhibitors were screened for their ability to inhibit activity of the enzyme. To better understand the mechanism of inhibition, changes in solvent accessibility of the protein were monitored in the presence of inhibitors and DNA. Previously, probing was performed using a combination of chemical modification and mass spectrometry. A complementary method of studying these interactions is to subject a protein to limited proteolysis under different conditions and to compare cleavage patterns. Previous studies using the metal-chelate complexes iron-ethylenediaminetetraacetic acid (Fe-EDTA) and copper-phenanthroline (Cu[OP]2) as proteases were successful in mapping interactions with other protein-ligand complexes. In this study we show that changes in proteolytic patterns of RT occur in the presence of DNA and inhibitors and show that subunit specific changes can be observed when an immunogenic tag is attached to the N-terminus of that subunit.

POSTER 21

ANTIVIRAL PROPERTIES OF ALPHA-P-BORANO-2',3'-DIDEOXYNUCLEOTIDE ANALOGUES

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2',3'-Dideoxynucleosides (ddNs) are widely used in the clinical treatment of AIDS. Most antiviral nucleosides require stepwise phosphorylation to the respective 2',3'-dideoxynucleoside triphosphates (ddNTPs), which inhibit the viral DNA synthesis. However, the emergence of HIV-1 reverse transcriptase-dependent drug resistance limits the effectiveness of treatment by ddNs. The alpha-P-borano-nucleotide analogues show several unique physico-chemical and biological properties: (i) Enzymatic studies indicate that the Rp- isomer of alpha-P-borano-ddNDPs is a better substrate for cellular NDP kinase than the parent ddNDP. (ii) Neither isomer of the alpha-P-borano-ddNDPs is a substrate for mammalian pyruvate kinase and shows very poor inhibitory properties to this enzyme. (iii) The Rp-(alpha-P-borano)-ddNTP isomers are better inhibitors of drug- and multidrug-resistant viral reverse transcriptases and are poor substrates for DNA-dependent DNA polymerases. (iv) After incorporation into viral DNA the borano-ddNMP residues are more resistant to ATP-dependent removal from viral DNA than parent ddNTPs. To by-pass inefficient phosphorylation of the antiviral nucleosides several prodrugs of alpha-P-borano-nucleotide analogues have been synthesized. Selective inhibition of drug- and multi-drug resistant viral RTs and poorer inhibition of intracellular kinases and DNA polymerases by the alpha-P-borano-nucleotide analogues suggest a new approach to the design of more powerful antiviral drugs.

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POSTER 22

OPTIMIZATION OF MULTICODE-RTX® REAL-TIME PCR SYSTEM FOR DETECTION OF SUBPOPULATIONS OF K65R AND M184V HIV-1 MUTANT VIRUSES

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Background: Standard genotyping analyses of plasma samples from HIV-1–infected patients only allow detection of drug-resistant mutants that constitute at least 20-30% of the viral population. In this study we improved the MultiCode-RTx Real-Time PCR (RTx) platform to allow highly sensitive testing for K65R and M184V RT mutants in clinical samples containing polymorphisms and low viral loads.

Methods: RT-PCR products containing the protease and RT region of HIV-1 from plasma samples were analyzed by the RTx utilizing K65 and M184 loci-specific primers. We modified this assay by including “curative” primers that correct polymorphisms which could interfere with the detection of mutant viruses. Single genome sequence analysis (SGS) of patient samples was also performed by diluting cDNA to a single copy followed by PCR and sequencing analysis.

Results: Plasma samples from 34 treatment-naïve HIV-1 patients were analyzed to determine a background level amplification for K65R or M184V mutants. We found that all baseline samples did not exceed the background of 0.5% for K65R detection. To verify quantification of the K65R mutation by RTx, we performed SGS for patient plasma samples that subsequently developed full K65R. Resultant quantification by RTx and SGS were in high agreement demonstrating 2-6% K65R in these samples which was not detectable by population sequencing. In the case of M184V, 33 out of 34 samples did not exceed the 0.1% background for baseline samples and one sample showed presence of M184V at 0.13 %. Sensitivity of M184V detection was verified by finding low-levels of this mutation (2-18%) in plasma samples from treatment-experienced patients who subsequently developed M184V.

Conclusions: The MultiCode-RTx RT-PCR system represents a sensitive and specific platform technology for use in the early detection of low-levels of drug-resistant viruses. Detection and quantification of low-frequency K65R mutation by this method was in concordance with SGS.

POSTER 23

MULTIFACETED INHIBITORY EFFECT OF RIBAVIRIN ON HANTAAN VIRUS REPLICATION

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Ribavirin inhibits a broad spectrum of RNA viruses; remarkably, it is one of the few antiviral drugs identified with such a broad activity. Several different mechanisms of action for ribavirin have been reported but it has not been clear which mechanism predominates to inhibit production of Hantaan virus (HTNV). Herein we investigated various aspects of the mechanism of action of ribavirin on HTNV. In a dose response assay of ribavirin and mycophenolic acid, we did not observe the correlation between GTP repression and antiviral effect of ribavirin. These results suggest that antiviral activity of ribavirin was not due to GTP repression. Furthermore, the addition of guanosine in the cell culture medium did not abolish the antiviral effect of ribavirin as we observed with MPA. By measuring the specific infectivity of HTNV, we found that ribavirin decreased specific infectivity along with increase of G to A or C to U mutations in dose dependent manners. The mutation frequency of HTNV was 6.7 mutations per 10,000 nt, which suggests that HTNV replicates very near at error catastrophe. Our studies suggest that the effect of ribavirin on HTNV is pleiotropic and targets both cellular metabolism and viral replication. We hypothesize that the mechanism is primarily by the error catastrophe with high mutation rate in normal population and moreover, that ribavirin incorporation can be enhanced by GTP depression.

POSTER 24

HIGH-THROUGHPUT SCREENING FOR INTEGRASE INHIBITORS USING A NOVEL ELECTROCHEMOLUMINESCENCE ASSAY

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Inhibitors of reverse transcriptase and protease are efficient anti-HIV drugs. Integrase (the 3rd retroviral enzyme) is also a rational drug target because it is required for viral replication and does not have a known cellular equivalent. Two integrase inhibitors have recently started clinical trials. Our recent studies demonstrate that integrase inhibitors can be screened using a novel high-throughput assay based on electrochemiluminescence (BioVeris®). Using recombinant HIV-1 integrase, we have now screened the 3161 compounds from the three NCI Developmental Therapeutics Program (DTP) chemical libraries. A total of 123 compounds are inhibitory at 10 μM (3.9% hit ratio). These 123 lead compounds are being examined in gel-based assays to evaluate their potency and selectivity for particular steps of the integrase reactions. The compounds are prioritized in collaboration with our colleagues from the NCI-CCR Laboratory of Medicinal Chemistry, the HIV Drug Resistance Program and the HIV and AIDS Malignancy Branch. Highest priority compounds are examined for mechanism of action, antiviral activity and structure-activity relationship with the aim of optimizing the drugs for medicinal development. A patent application for tropolones as anti-integrase inhibitors and anti-HIV drugs has been filed. We also discovered several tetracyclines active against integrase and with anti-HIV activity (recent patent from The Johns Hopkins University). Additional compounds with structures differing from known inhibitors are being pursued with the aim of providing novel anti-integrase drugs that can be patented and developed as new therapeutics against HIV and AIDS. The novel compounds will also be added to our pharmacophore collection and used to select compounds to be screened from other chemical libraries (ChemNavigator and NIH Roadmap initiative).

POSTER 25

LIS1 INDUCES HIV-1 TAT-MEDIATED TRANSCRIPTION

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Lissencephaly is a severe brain malformation in part caused by mutations in the LIS1 gene. LIS1 interacts with microtubule-associated proteins, and enhances transport of microtubule fragments. We show here that LIS1 is part of large protein complex associated with HIV-1 Tat, that also contains CDK Activating Kinase, CAK. LIS1 but not CAK associates with Tat through WD40 domains of LIS1, including domain 5. The effect of LIS1 on Tat-mediated transcription of HIV-1 LTR was analyzed in transient transfection assays using cell-permeable domains of LIS1. The WD5 but not the N-terminal domain of LIS1 increases Tat-dependent HIV-1 transcription in HEK293 cells. Overexpression of LIS1 in cells also showed an increase in Tat-induced transcription from the HIV-1 LTR. We noticed that the effect of LIS1 was similar to the effect of okadaic acid, an inhibitor of protein phosphatase 2A (PP2A), which induces HIV-1 transcription by upregulating the level of NF-κB. We hypothesized that WD40 domains of LIS1 resemble a B-subunit of PP2A, which also contains WD40 repeats. Previously, HIV-1 replication has been shown to be sensitive to the enzymatic activity of PP2A. Therefore we analyzed the effect of LIS1 on the activity of PP2A in vitro. We show that LIS1 and its isolated WD5 domain but not the N-terminal domain of LIS1, which lacks WD repeats, blocks PP2A activity in vitro. This finding may identify LIS1 as a yet unrecognized regulatory subunit of PP2A. We hypothesize that inhibition of PP2A by LIS1 upregulates the level of NF-κB and induces HIV-1 transcription.

POSTER 26

COMPUTATIONAL PREDICTION OF HIV-1 REVERSE TRANSCRIPTASE DRUG RESISTANCE MUTATIONS

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Background: In this exercise we identify a novel computational approach to predict effects of drug resistance based on an enzyme's amino acid mutations. We used a computational geometry method, in conjunction with Support Vector Machine (SVM) and Random Forest supervised machine learning algorithms, to infer mutational drug resistance patterns of reverse transcriptase (RT).

Results: Although there is limited availability of mutational drug resistance data for RT, we were able to achieve accurate discrimination between mutational patterns that either confer drug sensitivity or lead to drug resistance by combining novel computational techniques. Control sets and variable training sets were used as a reference to evaluate the quality of predictions. Accuracy rates for drug resistance based on known mutations in computational runs were close to 90% for some runs when using Random Forest and SVM algorithms.

Discussion: Based on known mutant RT genotypes, we can evaluate levels of effectiveness of several Non-Nucleotide RT Inhibitors (NNRTI) available for HIV treatment using geometric tessellation in conjunction with known computational machine learning algorithms.

POSTER 27

HIV STRUCTURAL DATABASE: A STRUCTURAL RESOURCE FOR INDUSTRIAL AND ACADEMIC RESEARCHERS TO FACILITATE RATIONAL DRUG DESIGN

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Federal agencies, academia, and industry have invested heavily in the development of structural protein and chemical databases. A hurdle to realizing the benefits of this information is that the data are distributed over several public and private archives, leading to issues of interoperability. Technical challenges relating to the integration of data weaken the appeal of bioinformatics databases. The issue, however, creates an ongoing struggle in the broader life science research setting and has to be resolved in order to provide maximum possible value and eliminate incompatibilities. To maximize the return on this investment, the information must be widely accessible by a broad community of scientific researchers. NIST, in collaboration with NCI, NIAID, and Rutgers University, has been working on the development of an integrated 2-D and 3-D structural data resource for AIDS (http://xpdb.nist.gov/hivsdb/hivsdb.html). HIV-Structural-DB has obtained structural data and related information for HIV proteins from various sources and compiled these data for efficient retrieval. A novel technique combining chemical taxonomy, data-tree, and Chem-BLAST is used to analyze, annotate, integrate, and set customized queries to extract both 2-D and 3-D structural information, empowering the research community to more effectively understand and use structural information towards HIV research. The data annotation and distribution techniques used in this database lay the foundation for semantic web technology for biological data with special emphasis on rational drug design by the mix and match approach. The data, annotation, and distribution techniques developed and applied at NIST will be presented.

POSTER 28

EXAMINING TY3 POLYPURINE TRACT STRUCTURE AND FUNCTION BY NUCLEOSIDE ANALOG INTERFERENCE

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We have combined nucleoside analog interference with chemical footprinting, thermal denaturation, and biochemical studies to examine recognition of the polypurine tract (PPT) primer of the *Saccharomyces cerevisiae* LTR-retrotransposon Ty3 by its cognate reverse transcriptase (RT). Locked nucleic acid (LNA) analogs, which constrain sugar ring geometry, were introduced pairwise throughout the PPT (-) DNA template. In addition, abasic tetrahydrofuran (THF) linkages, which lack the nucleobase while preserving the sugar-phosphate backbone, were introduced throughout the (-) strand DNA template and (+) strand RNA primer. Subsequently, the stability of substituted duplexes and their ability to bind Ty3 RT, along with their effect on (a) selection of the (+) strand primer and (b) its extension into (+) strand DNA, were examined. Collectively, our data suggests that both the 5' and 3' portions of the PPT-containing RNA/DNA hybrid are sensitive to nucleoside analog substitution, while the intervening region can be modified without altering cleavage specificity. Achieving similar phenotype with nucleoside analogs that have different effects on duplex geometry reveals structural features are important mediators of PPT recognition by Ty3 RT. Finally, introducing abasic THF lesions around the scissile PPT/U3 junction indicates that template nucleobase -1 is dispensable for catalysis, while a primer nucleobase on either side of the junction is necessary.

POSTER 29

ANALYSIS OF HIV-1 DNA SYNTHESIS AND THE FATE OF REVERSE TRANSCRIPTS IN THE PRESENCE OF APOBEC3G IN *VIF*- AND *VPR*-DELETED VIRUSES

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Encapsidation of APOBEC3G, a host cytidine deaminase, into HIV-1 virions blocks virus replication by catalyzing the C-to-U deamination of newly synthesized reverse transcripts. The HIV-1 viral infectivity factor (*vif*) protein binds APOBEC3G and induces its proteasomal degradation, thereby preventing its packaging into nascent virions. It has been shown that the block to virus replication is due to the extensive G-to-A hypermutation. In addition, it has been hypothesized that degradation of the uracil-containing reverse transcripts by host DNA repair enzymes prior to integration may be critical for inhibition of viral replication. One such host enzyme is uracil DNA glycosylase (UNG-2), which removes uracil from DNA generating an abasic position which is proposed to result in viral DNA degradation by the host AP endonuclease. UNG-2 has been shown to interact with the HIV-1 viral protein R (*vpr*) and integrase. It is believed that the interaction of UNG-2 with the two viral proteins may be involved in its packaging into virions. However, a recent study showed that binding of *vpr* to UNG-2 and SMUG-1, another host uracil DNA glycosylase, induces proteasomal degradation of the host enzymes, thereby excluding them from being packaged into virions.

To gain further insight into the dynamics of HIV-1 DNA synthesis and the fate of reverse transcripts in the presence of APOBEC3G, we compared the amount of reverse transcripts as well as integrated proviruses in the presence and absence of *vif* and/or *vpr* using a real-time quantitative PCR assay. Our results show that the amount of both early and late reverse transcription products is reduced by 10-fold in *vif*-deleted as well as *vif/vpr*-deleted viruses in the presence of APOBEC3G compared to no APOBEC3G controls. However, APOBEC3G reduced the infectivity of *vif* and *vif/vpr*-deleted viruses by 90 and 140-fold respectively, suggesting that other factors such as hypermutation or a block in a post-DNA synthesis step in replication may contribute to the loss of infectivity, in addition to the reduction in viral DNA synthesis.

POSTER 30

STOICHIOMETRY OF THE ANTIVIRAL PROTEIN APOBEC3G IN HIV-1 VIRIONS

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A host cytidine deaminase, apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G), inhibits replication of human immunodeficiency virus type 1 (HIV-1) by incorporating into virions in the absence of the virally-encoded Vif protein (ΔVif HIV-1 virions) and causing G-to-A hypermutation. To elucidate the mechanism of antiretroviral function of APOBEC3G, we used three experimental approaches to determine the quantities of APOBEC3G that are incorporated into ΔVif HIV-1 particles. First, we used a previously described scintillation proximity assay to measure APOBEC3G enzymatic activities; second, we performed quantitative immunoblotting analysis to determine the amounts of APOBEC3G present in virions; and third, we performed high-pressure liquid chromatography analysis to determine the amounts of HIV-1 capsid and APOBEC3G in virions. Using these three approaches, we determined the molar ratios of APOBEC3G to HIV-1 capsid protein. We analyzed 293T cells transfected with various amounts of APOBEC3G expression plasmid and found that APOBEC3G incorporation into ΔVif virions was proportional to the level of expression in virus-producing cells. Analysis of the stoichiometry of APOBEC3G to HIV-1 CA in ΔVif virions produced from human primary $CD4^+$ T cells indicated that the molar ratio of Gag to APOBEC3G was approximately 400 ~ 830:1. Based on previous estimates of the stoichiometry of HIV-1 Gag in virions (1400–5000), we conclude that approximately 2–13 molecules of APOBEC3G are incorporated into ΔVif virions produced from human primary $CD4^+$ T cells. These results indicate that virion incorporation of only a few molecules of APOBEC3G may be sufficient to inhibit HIV-1 replication.

POSTER 31

THE CYTOPLASMIC LOCALIZATION OF APOBEC3F AND APOBEC3G IS INDEPENDENT OF CRM1, UNLIKE THEIR HOMOLOGS AID AND APOBEC1

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APOBEC family members APOBEC1 and activation-induced deaminase (AID) are actively exported from the nucleus via the CRM1 (exportin-1) pathway. Like AID, APOBEC3F (A3F) and APOBEC3G (A3G) appear to localize predominantly or exclusively to the cytoplasm. Furthermore, A3F and A3G contain putative leucine-rich nuclear export signals at their carboxyl-termini. For these reasons, we hypothesized that A3F and A3G, like their family members, might be regulated by CRM1. To test this hypothesis, we took two approaches. First, we took advantage of the specific CRM1 export inhibitor, leptomycin B. After leptomycin B treatment, GFP fluorescence began to accumulate in the nucleus of 293T cells expressing AID-GFP but not in cells expressing A3F-GFP or A3G-GFP. Second, we created A3F-GFP and A3G-GFP constructs that lacked the putative nuclear export signals. GFP fluorescence in cells expressing these deletion constructs remained cytoplasmic. Thus, we conclude that A3F and A3G are not exported via the CRM1 pathway, and their distinct cytoplasmic localization must be attributable to other factors.

POSTER 32

INTERFERON REGULATED TRANSCRIPTION OF APOBEC3G AND APOBEC3F

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APOBEC3G and 3F are cytidine deaminases that can hypermutate HIV genomes during reverse transcription, and reduce infectivity of HIV and HBV. HIV vif promotes their degradation, thereby limiting the amount packaged in the virion. If the amount of APOBEC in the HIV-producer cell outweighs the activity of vif, APOBEC can still be packaged and affect virion infectivity. Therefore, we seek to identify factors that increase the levels of APOBEC3G / 3F in HIV-producer cells. Potential binding sites for interferon regulated transcription factors ISRE and IRF are identifiable upstream of the APOBEC3G and 3F start codons (TransFac Database). To investigate if transcription of APOBEC3G / 3F can be regulated by interferons, primary human CD4+ T cells and HepG2 hepatocellular carcinoma cells were treated with Type I and Type II interferons. Transcription was not increased in CD4+ lymphocytes by either interferon αA/D or interferon γ. TCR and PHA stimulation each increased APOBEC3G and 3F transcription. Interferon γ, but not interferon αA/D, caused an 8-fold maximal increase of both APOBEC3G and 3F RNA at 24 hours after treatment of HepG2 hepatocellular carcinoma cells. VSV-G pseudotyped, GFP-expressing HIV-1 virions produced from interferon γ-treated HepG2 cells had reduced infectivity relative to virions produced from untreated cells. Although vif-deleted reporter viruses were less infectious, the interferon γ effect was seen whether vif was functional or not. Sequence analyses and siRNA knockdowns of APOBEC3G and 3F will confirm if the interferon γ effect on virion infectivity is caused by increased APOBEC3G / 3F expression. Although interferon treatments did not increase transcription in primary CD4+ lymphocytes, transcriptional regulation by interferon γ was demonstrated in HepG2 cells. We are continuing to investigate interferon regulation of APOBEC3G / 3F transcription in subsets of CD4+ lymphocytes and other primary HIV producer cells.

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POSTER 33

LOW RESOLUTION STRUCTURAL MAPPING OF HIV-1 VIF USING MASS SPECTROMETRY

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The role of HIV-1 Vif on infectivity and its interactions with APOBEC3G, HIV-1 Gag, HIV-1 Protease and SOCS are current areas of extensive investigation. Vif oligomerization appears to be important for these interactions. It has been previously reported that the oligomerization domain of Vif is amino acid residues 151-164, and if this region was deleted or mutated there was a significant reduction in Vif's function. Chemical cross-linking along with 18O labeling and mass spectrometric analyses was used to identify domains involved in Vif oligomerization and to obtain further insight into Vif's structural characteristics. Cross-linking showed evidence of a monomer, dimer and trimer species via denaturing gel in addition to a tetramer via western blot. Between the four samples – noncross-linked, monomer, dimer, and trimer – unique linear peptides and non-contiguous cross-linked peptides were identified. A few of these peptides were conserved among the various oligomers, and others were not. This suggested oligomerization induced conformational changes affecting the accessibility to a few of the cleavage sites. A particularly interesting fragment observed only in noncross-linked and in monomer was a fragment containing residues 159-173 which contain the previously proposed oligomerization domain, 151-164. In addition, LCMS data collected identified amino acids 158-168 to be key residues involved in dimerization. Therefore, we propose that Vif has a specific and unique tertiary structure along with specific oligomeric forms, suggesting that the protein undergoes a conformational change that exposes different binding interfaces to form higher order oligomers. In the absence of crystallographic data, pursuing our low resolution structural analyses will provide momentum for Vif-targeted structure based drug designs.

POSTER 34

ANTIVIRAL ACTIVITY OF AMPHOTERICIN B METHYL ESTER (AME): ISOLATION OF AME-RESISTANT MUTANTS

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Previous studies from our lab demonstrated that membrane cholesterol plays an important role in HIV-1 assembly, release, and infectivity. Amphotericin B methyl ester (AME), a water-soluble, relatively non-toxic derivative of the polyene antibiotic amphotericin B (AB), is a cholesterol-binding compound. Antiviral activity of AB and its derivatives against a range of enveloped viruses has been reported; however, little is known about the mechanism of inhibition. To investigate the effect of AME on the HIV-1 replication cycle and to evaluate the potential for AME as an antiretroviral, we determined the effect of AME on HIV-1 replication in various T-cell lines and in primary cells. We observed that virus replication was potently inhibited. We also found that AME profoundly impaired HIV-1 infectivity in single-cycle assays in CD4+ HeLa-derived TZM cells. To investigate the effect of AME on the late stages of the virus replication cycle, we performed virus release assays in HeLa and Jurkat cells. We observed a ~5-fold decrease in virus particle production, with no significant effect on Gag binding to the plasma membrane, Gag association with lipid rafts, or Gag multimerization. To study further the antiviral properties of AME, we selected for AME-resistant virus in Jurkat T-cells. The mutations responsible for AME resistance, P203L and S205L, mapped to an endocytosis motif in the cytoplasmic tail of gp41. Virus replication and single-cycle infectivity assays confirmed that the P203L and S205L substitutions conferred AME resistance. Interestingly, truncation of the gp41 cytoplasmic tail of either HIV-1 or SIV also conferred resistance. This study sheds light on the target and mechanism of action of AME and provides support for the concept that cholesterol-binding compounds should be pursued as antiretrovirals.

POSTER 35

ROLE OF AIP1 IN HIV-1 GAG PROCESSING AND VIRUS RELEASE

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The p6 domain of HIV-1 Gag contains sequence(s) that are critical for virus particle budding and release. A Pro-Thr-Ala-Pro (PTAP) motif in p6 serves as the major “late” domain of HIV-1 and promotes particle release by binding the endosomal sorting factor Tsg101. However, it has also been reported that a region of HIV-1 p6 distinct from PTAP interacts with a second factor known as AIP1. While it has been clearly demonstrated that AIP1 is critical for the release of equine infectious anemia virus, the role of AIP1 in promoting HIV-1 particle production is not well understood. To evaluate the role of AIP1 in HIV-1 replication, we introduced mutations in the AIP1 binding site of p6 and examined the effects on virus assembly and release in HeLa cells. While AIP1 binding site mutations did not reduce levels of virus egress, we observed elevated levels of Gag processing intermediates for the mutants, thus suggesting a processing defect. To examine the importance of the AIP1 binding site in physiologically relevant cell types, the replication of AIP1 binding site mutants was studied in T-cell lines and in primary PBLs. The mutants showed defective replication compared to the WT. Release of WT and mutant virus from Jurkat cells was subsequently measured to characterize the replication defect. Preliminary results indicate that while the levels of mutant and WT virus release were comparable, the mutants again displayed defective Gag processing. Thus, binding of HIV-1 Gag to AIP1, while not important for promoting efficient HIV-1 particle release, appears to regulate proper Gag processing not only in HeLa cells but also in T cells. Current studies are aimed at deciphering the mechanism by which AIP1 binding regulates Gag processing and understanding the functional importance of the AIP1 binding site in HIV-1 replication.

POSTER 36

EFFECT OF HIV-1 PROTEASE DISTAL MUTATIONS ON DRUG RESISTANCE MEDIATED BY THE CHANGES OF ITS MOLECULAR DYNAMICS

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Drug resistance of HIV-1 protease is a complex effect caused by mutations at multiple sites within the protease structure. It was assumed that the primary source of the resistance are mutations of active site residues that are in direct contact with inhibitors. Recently, a crucial role was shown for distal mutations in conferring drug resistance to the enzyme, however. This suggests a necessity to explore the mechanisms of distal mutations in order to assist discovery of resistance evading inhibitors of the next generation.

Assuming that the contribution of distal mutations to the resistance profile could be mediated by conformational dynamics we modelled L10I, L90M, L10I/L90M, A71V/G73S/I93L (triple) and E35Q/M36I/S37D/R57K (quatro) mutants of HIV-1 protease. These mutants were subjected to 10ns molecular dynamics simulations and compared with wild type protease using the Essential Dynamics analysis protocol.

Our results reveal that mutant L10I and triple were shown to stabilize the closed conformation and improve the overall stability of HIV-1 protease. Mutation L10I alters the flexibility of the N-terminal loop that, in turn, affects the stability within the dimerization domain of the protease. Such flexibility restrictions propagate to the core and flap domains through the correlated motions of the enzyme. The triple mutant, on the other hand, alters flexibility at the interface of the core and dimerization domains that is necessary for the flap opening event. The L90M mutant disturbs the unity of correlated motions of the dimerization domain that result in decreased stability of the protease dimer molecule. Sequentially, the mistuning of the domain correlated motions is observed. Four mutations of the quatro mutant have not exhibited any effect on HIV-1 protease molecule dynamics suggesting that the distal mutations of the flap domains may become important upon either ligand binding or in longer timescales.

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POSTER 37

STRUCTURAL AND BIOCHEMICAL ANALYSIS OF THE MOLECULAR EVOLUTION: THE CASE OF NELFINAVIR AND HIV PROTEASE

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HIV protease is one of the key targets of anti-HIV treatment. Resistance development represents a serious obstacle to successful therapy. In this study, we investigated the parameters driving nelfinavir resistance along two separate evolutionary pathways. It has been shown that in most patients the resistance to nelfinavir evolves by selecting the D30N mutation in the viral protease. However, in minority of cases, less specific L90M mutation is initially acquired. Viral species with both these substitutions are very rare and a major loss of replicative capacity for a mutant clone carrying both D30N and L90M substitutions was described. To analyse structural and biochemical basis driving these evolutionary pathways, we engineered eight HIV PR mutants harbouring D30N, N88D, L90M and A71V mutations alone and in combination. We expressed and purified them in bacteria, determined their catalytic efficiencies in vitro and measured the Ki values for nelfinavir and a panel of selected PIs including a pseudopeptide inhibitor QF34 designed previously in our laboratory. From these values, we were able to calculate the vitality of individual proteases as a measure of their relative resistance, and correlate them with the replicative capacity of corresponding recombinant viruses (Perrin and Mammano, J. Virol. 77, 10172, 2003). In order to elucidate the structural basis for resistance and mutual exclusivity of individual pathways, we crystallized and analyzed the 3D-structures of four individual mutated proteases (D30N, D30N/L90M, D30N/A71V, and D30N/N88D) in complex with nelfinavir at resolution ranging from 2.4 – 1.85 Å. We show that subtle structural changes in mutated PRs translate to significant variations of enzymatic activities and dramatic differences in replication capacity of corresponding recombinant viruses.

POSTER 38

MOBILITY OF HIV-1 P55 IN LIVING CELLS USING FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING AND A PHOTOACTIVATABLE GFP

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One of the key aspects of viral assembly is the mobility of Gag molecules. To gain insights into the dynamic aspects of HIV assembly we have utilized two approaches that allow the direct analysis of the mobility p55 Gag tagged with fluorescent protein derivatives in living cells: Fluorescence Recovery After Photobleaching (FRAP) and photoactivation. Using these methods, we have been able to characterize Gag-GFP mobility in live cells. Also, to gain insights into the movement of potential intermediates of the assembly process we have also analyzed the mobility of truncation Gag mutants and a myristoyl-deficient Gag mutant. We have found that unassembled Gag-GFP is highly mobile in living cells. We find that all derivatives except for the MACA truncation mutant have similar rates of mobility.

We normalized data produced from these experiments to two different parameters, the mobile fraction (R) and diffusion time (τ_D). With the exception of VLPs, our results show that as the Gag truncation mutants get smaller in size, the larger the mobile fraction with MA having the largest and VLPs having the smallest. As expected, Gag-, MA-, and G2AGag-GFP fusion proteins all have similar τ_D values with MACA having the largest. Gag-GFP VLPs had no τ_D value, reflecting a complete lack of mobility. We also find that cholesterol depletion causes a reversible decrease in Gag-GFP mobility. Our results suggest that Gag mobility decreases with progression of the assembly process and that mobility is cholesterol-dependent.

POSTER 39

PROTEIN-PROTEIN INTERACTIONS OF GAG: EFFECTS OF DIMERIZING AND TRIMERIZING ZIPPER MOTIFS ON HIV-1 GAG PARTICLE ASSEMBLY

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It has been proposed that dimerization of Gag, the sole protein needed for formation of virus-like particles, is a necessity for efficient assembly and production of VLPs (Ma and Vogt, J. Virol. 76: 5452, 2002; and Johnson, et al., J. Virol. 76: 11177, 2002). Evidence supporting this comes from the fact that the NC domain can be replaced by a dimerizing leucine zipper (Zhang et al., J. Virol. 72: 1782, 1998; Johnson et al., J. Virol. 76: 11177, 2002; and Accola et al., J. Virol. 74: 5395, 2000), and that the minimum length of nucleic acid capable of supporting assembly by wild-type Gag is roughly enough to accommodate two Gag molecules (Ma and Vogt, J. Virol. 76: 5452, 2002). However, an alternate possibility is that a small oligomer of Gag is needed to "nucleate" assembly, and that a trimer might be as good or better as an assembly initiator than a dimer. Indeed, Accola et al. reported that NC could also be replaced by a trimerizing leucine zipper domain, and the levels of particle production were similar with the two constructs.

In an effort to probe this possibility, we have constructed Gag chimeras in which the NC domain has been replaced by either a dimerizing zipper (Z_{Leu}) or a trimerizing zipper (Z_{Ile}) (kind gift from H. Göttlinger). We confirmed the observation that Gag-Z_{Leu} and Gag-Z_{Ile} produce VLPs at a level very similar to wild-type Gag, and upon examination of VLPs produced in mammalian cells, we found that both zipper constructs assembled into very homogenous, spherical VLPs with a diameter ~ 125 nm. In fact, the dimensions of Gag-Z_{Leu} and Gag-Z_{Ile} VLPs were indistinguishable from one another and more uniform than those assembled from wild-type Gag. This finding suggests that either dimers or trimers of Gag can serve as intermediates in the assembly of VLPs. We are continuing to characterize assembly of these chimeric Gag proteins, both in mammalian cells and in a defined system *in vitro*.

POSTER 40

ROLE OF DILEUCINE-LIKE MOTIFS IN THE HIV-1 CAPSID IN VIRUS ASSEMBLY AND RELEASE

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The pathway of Gag targeting to either the plasma membrane or multivesicular bodies (MVBs) in different physiologically relevant cell types remains poorly defined. It has been postulated that the C-terminus of the HIV-1 capsid (CA) domain of Gag contains diLeu-like endocytosis motifs that promote the transport of Gag from the plasma membrane to MVBs. The aim of this study was to elucidate the importance of the proposed endocytosis motifs within HIV-1 capsid in virus assembly and release.

Analysis of the diLeu-like motif mutants IL-333,334AA and LL-321,322AA revealed that both exhibit a significant defect in virus release compared to WT Gag. Fluorescence microscopy studies indicated that the LL-321,322AA mutant failed to show surface punctate staining observed for WT Gag or the IL-333,334AA mutant. These results suggested the LL-321,322AA mutant possessed a defect in Gag multimerization and Gag membrane binding similar to that induced by mutation of residues W-184 and M-185 at the CA dimer interface. These results were confirmed by membrane flotation assays. The release defect of the IL-333,334AA mutant could be attributed mainly to CA protein misfolding; this mutant could be efficiently rescued by coexpression with WT Gag but displayed a novel pattern of CA processing products. Defective Gag multimerization for the LL-321,322AA mutant was indicated by its inability to be efficiently rescued by WT Gag. Finally, EM studies revealed that IL-333,334AA mutant virions are unusually large with aberrant/immature cores.

While these results do not exclude the possibility that the diLeu-like motifs within HIV-1 CA may interact with the cellular endocytic machinery, they suggest that the primary mechanism by which these mutations disrupt virus particle production involves major defects in Gag-Gag multimerization, CA stability, and membrane binding. The mutants described here will be valuable tools to understand the interplay between CA dimerization, Gag multimerization, membrane binding, virus assembly, and particle maturation.

POSTER 41

THE ROLE OF ESCRT-I COMPONENTS IN ENDOSOMAL SORTING AND HIV-1 RELEASE

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The cellular proteins Tsg101, Vps28 and Vps37 constitute a high molecular weight complex (ESCRT-I) involved in sorting ubiquitinated cargo proteins into the endosomal pathway and in the biogenesis of multivesicular bodies (MVBs). HIV-1 and other retroviruses have usurped the normal function of ESCRT-I and associated machinery to promote virus release from cells. In the case of HIV-1, a direct binding occurs between Gag and Tsg101; this interaction connects HIV-1 Gag to the MVB machinery. We have previously shown that overexpression of the N-terminal fragment of Tsg101 (TSG-5') inhibits virus release by directly binding the p6 domain of Gag. Overexpression of full-length Tsg101 (TSG-F) also inhibits HIV-1 release, but unlike TSG-5', TSG-F-mediated inhibition occurs not by direct Gag/Tsg101 binding but by global disruption of the endosomal sorting machinery. This endosomal sorting defect leads to intracellular accumulation of the EGF receptor and formation of enlarged, aberrant endosomes in TSG-F-overexpressing cells. To characterize the mechanism by which TSG-F overexpression disrupts endosomal sorting and virus release, we constructed a series of C-terminal Tsg101 truncation mutants and analyzed their effect on Tsg101 localization, endosomal sorting, and virus release. The region critical for the formation of swollen endosomes was mapped to Tsg101 residues 355 to 365 near the C-terminus of the protein. We have also evaluated the ability of Tsg101 mutants defective in binding to Vps28 and Vps37 to disrupt endosomal sorting and virus release. As an extension of this approach, we have determined the impact of overexpressing WT and mutant forms of Vps28 and Vps37 on sorting and HIV-1 budding. The results will help elucidate the complex interplay between ESCRT-I components, the endosomal sorting pathway, and retrovirus release.

POSTER 42

FIV RELEASE REQUIRES ENDOSOMAL SORTING MACHINERY AND A PRO-SER-ALA-PRO (PSAP) MOTIF NEAR THE C-TERMINUS OF GAG

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Short peptide motifs within retroviral Gag precursor proteins are essential for efficient release of assembled virions from infected cells and are therefore referred to as “late domains”. These domains may mimic sequences found in cellular proteins that interact with endosomal sorting complexes required for transport (ESCRT-I, II, III) first described in yeast vacuolar protein sorting (Vps) mutants. Normally, interaction of ubiquitinated cargo with ESCRT complexes promotes budding of intracellular vesicles into late endosomes; in infected cells, late domains within Gag hijack ESCRT machinery to facilitate virus release. The PTAP motif of HIV-1, for example, has been shown to interact with the ESCRT-I component Tsg101. HIV-1 release is severely restricted upon expression of the N-terminal fragment of Tsg101 (TSG-5’), which contains a PTAP binding site. The non-primate lentivirus FIV contains a similar motif (PSAP) within Gag, which we predicted would make FIV release sensitive to inhibition by TSG-5’. This prediction was borne out in both HeLa and CrFK cells, as determined by biochemical, virological, and EM analyses. We also demonstrated by site-directed mutagenesis that the PSAP motif is essential for FIV replication and release from CrFK and release from HeLa cells. It has been suggested that HIV-1 may possess a secondary late domain that binds AIP-1, which facilitates association with ESCRT I and III. Similarly, a putative secondary late domain motif that is C-terminal to PSAP in FIV Gag bears resemblance to an AIP-1 binding site. By site-directed mutagenesis, we have found that FIV does not rely on this motif for virus replication or release in either CrFK or HeLa cells. Further characterization of late domain usage by FIV in feline lymphocytes is underway. The results of these studies will elucidate the viral and cellular determinants of FIV release.

POSTER 43

UBIQUITIN PLAYS A ROLE IN BUDDING OF HTLV-1

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Many accessory and some structural retroviral proteins consist of aggregates of peptide motifs which function binding sites to recruit and redirect cellular proteins to perform functions necessary in the viral lifecycle. The motifs (P(S/T)AP, PPPY, YPDL) of the late domain (LD) divert the cellular multivesicular bodies (MVB) biogenesis pathway to function as the retroviral budding machinery. The motifs are similar to those used by components of the MVB pathway to connect to each other. MVBs are part of the late endosomal compartment of the cell used to degrade activated cell surface receptors, which are marked for entry into the pathway by modification with one to four ubiquitins. Most retroviral proteins carrying the LD are also modified by low-level ubiquitination. The role of this modification – another recruitment signal for the MVB components or just a bystander effect – is still debated. The MA of Human T-cell Leukemia Virus type 1 (HTLV-1) contains PPPY and PTAP motifs, and in addition is more heavily ubiquitinated than the LD-peptides of other retroviruses. To assess the role of the ubiquitin modification in HTLV-1 budding, we mutated the four lysine residues in MA and showed that only K74 functions as a substrate for mono- and di-ubiquitin addition in MA. HTLV-1 expression constructs carrying the K74R mutation were impaired in budding and showed the typical accumulation of viral proteins under the cell membrane by transmission electron microscopy. However, the late domain effect was not as pronounced as that observed for mutants with a change in the PPPY motif. Analysis of the Gag precursor and CA and NC of the K74R mutant HTLV-1 showed that targets in the NC part of the protein are ubiquitinated. The position and role of this modification for the limited budding of the K74R mutant is being currently investigated.

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